

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 7/01, 15/34, 15/87		A1	(11) International Publication Number: WO 99/02658 (43) International Publication Date: 21 January 1999 (21.01.99)
(21) International Application Number: PCT/US98/14239		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 8 July 1998 (08.07.98)			
(30) Priority Data: 60/088,993 9 July 1997 (09.07.97)		US	Published <i>With international search report.</i>
(71) Applicant: SAINT LOUIS UNIVERSITY [US/US]; 221 N. Grant, St.Louis, MO 63103 (US).			
(72) Inventor: WOLD, William, S., M.; 1609 Adgers Wharf, Chesterfield, MO 63017 (US).			
(74) Agents: HOLLAND, Donald, R. et al.; Howell & Haferkamp, L.C., Suite 1400, 7733 Forsyth Boulevard, St. Louis, MO 63105 (US).			

(54) Title: INHIBITING APOPTOSIS WITH ADENOVIRUS RID PROTEIN

(57) Abstract

A method for inhibiting apoptosis of a cell expressing a death receptor of the TNFR family is disclosed. The method involves treating the cell with a Receptor Internalization and Degradation (RID) protein complex containing RID α (10.4K) and RID β (14.5K) proteins encoded by the E3 region of adenovirus. The cell can be treated by administering to the cell a polynucleotide expressing the RID complex or by administering to the cell a composition containing the RID complex. Compositions containing a RID complex are also disclosed. The compositions and method are useful in the treatment of cancer, degenerative and immune disorders, as well as in promoting survival of tissue transplants. An adenovirus vector for delivering the RID complex to cells is also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

Inhibiting Apoptosis with Adenovirus RID ProteinReference to Government Grant

This invention was made with government support under Grant Number RO1 CA58538. The government has certain rights in this invention.

5 Related Application

This application claims priority to U.S. Provisional Application serial number 60/088,993, filed July 9, 1997, which is incorporated herein in its entirety by reference.

Background of the Invention

10 (1) Field of the Invention

This invention relates generally to the regulation of apoptosis and, more particularly, to a method for inhibiting apoptosis using the Adenovirus RID protein and to applications of this method, including promoting survival of tissue transplants, treating autoimmune disease, and promoting tumor destruction in cancer patients.

15 (2) Description of the Related Art

Apoptosis, or programmed cell death, plays a fundamental role in regulation of the immune system. For review, see White, E. *Genes & Development* 10:1-15, 1996; van Parij, L. and Abbas, A.K., *Curr. Opin. Immunol.* 8:355-361, 1996; Nagata, S., *Cell* 88:355-365, 1997. In recent years researchers have shown that some members of the tumor necrosis factor 20 (TNF) family of cytokines can induce apoptosis by binding to their specific receptors on

target cells. Nagata, *supra*; Baker , S.J. and Reddy, E.P., *Oncogene* 12:1-9, 1996. The receptors for the TNF family of cytokines belong to a family of proteins referred to as the TNFR family, which is characterized by an extracellular domain of highly conserved cysteine residues contained in cysteine-rich pseudorepeats (Chaudhary et al., *Immunity* 7:821-830, 1997). In addition, several members of the TNFR family possess a conserved cytoplasmic domain of approximately 80 amino acids called the death domain, which functions to initiate an intracellular apoptotic signaling cascade upon binding of the appropriate cytokine. (See Chaudhary et al., *supra*; Walczak et al., *EMBO J.* 16:5386-5397, 1997.) TNFR proteins containing death domains comprise a death receptor subfamily which includes: TNFR1 (Tartiglia et al., *Cell* 74:845-853, 1993); Fas (also called CD95 and Apo-1) (Itoh and Nagata, *J.Biol. Chem.* 268:10932-10937, 1993); death receptor 3 (DR3, also called TRAMP, Apo-3, Wsl-1, and LARD) (Chinnaiyan et al., *Science* 274:990-992, 1996; Kiston et al., *Nature* 384:372-375, 1996); TRAIL-R1 (also known as DR4) (Pan et al., *Science* 276:111-113, 1997); and TRAIL-R2 (also called DR5) (Pan et al., *Science* 277:815-818, 1997). The death domains of these proteins are shown in Figure 1.

Fas, the most studied death receptor, is expressed on the surface of most cell types, including epithelial cells, fibroblasts, T and B cells, liver hepatocytes and some tumor cells (Nagata, *Nature Medicine* 2:1306-1307, 1996; French et al., *Nature Medicine* 3:387-388, 1997). However, FasL is primarily expressed by activated leukocytes of the immune system, including cytotoxic T lymphocytes (CTL's) and natural killer (NK) cells (Nagata, *Cell*, *supra*). It is believed that the Fas ligand (FasL) plays a role in the immune response of these cells to induce apoptosis in target cells expressing Fas. Such target cells include virus-infected cells and tumor cells. On the other hand, leukocytes also express Fas, which can result in down regulation of the immune response due to activated leukocytes killing each other (Nagata, *Cell*, *supra*).

Recently, it was discovered that FasL is also expressed in immune-privileged sites such as the eye chamber, parts of the nervous system, and testis and it is believed that any activated leukocytes entering such sites are immediately killed through the FasL-Fas apoptotic pathway, thereby preventing a potentially crippling immune response (Nagata, *Cell*, *supra*). This finding could potentially be applied to preventing transplant rejection and, indeed, one group has reported that islet allografts were protected from immune rejection by cotransplantation with syngeneic myoblasts expressing functional FasL (Lau et al., *Science* 273:109-112, 1996).

The discovery of FasL expression in immune-privileged sites led a number of groups to examine whether the means by which tumor cells avoid destruction is through expression of FasL. A number of tumor cell types were subsequently reported to constitutively express

FasL, including lymphoma and leukemia cells (Tanake, et al., *Nature Med.* 2:317-322, 1996) various nonlymphoid carcinoma cells, including colon cancer (O'Connell, et al., *J. Exp. Med.* 184:1075-1082, 1996), hepatocellular carcinoma (Strand et al., *Nature Med.* 2:1361-1366, 1996) and melanoma (Hahne et al., *Science* 274:1363-1366, 1996). As a result of expressing 5 FasL, many tumor cells have the ability to kill attacking CTL and NK cells thereby reducing the immune response against the tumor. In addition, it has been reported that some types of tumors become resistant to Fas-mediated apoptosis, either by downregulation of Fas expression or by other unknown mechanisms, and thereby avoid being killed by the infiltrating leukocytes (Nagata, *Nat. Med.*, *supra*; Strand et al., *supra*; Hahne et al., *supra*).
10 Because alterations in Fas-FasL regulation, including upregulation of FasL expression and downregulation of Fas expression, may be involved in tumor cells avoiding destruction by the immune system, it would be desirable to devise an approach that would reduce the effect of such changes in Fas-FasL regulation. In one such approach it was recently reported that the anti-cancer drug doxorubicin enhances expression of both Fas and Fasl in tumor cells (Friesen
15 et al., *Nature Med.* 2:574-577, 1996).

Recent reports have associated other disease states with dysfunction of the Fas system, including hypereosinophilic syndromes in humans (Lenardo et al., *J. Exp. Med.* 183:721-724, 1996), hepatitis (Kondo et al., *Nat. Med.* 3:409-413, 1997) and the autoimmune disease Hashimoto's thyroiditis (HT) (Giordano et al., *Science* 175:960-963, 1997).
20 Consequently, it has been suggested that inappropriate upregulation of Fas may be a causal factor in other autoimmune diseases involving tissues which constitutively express FasL (French et al., *supra*).

Human adenoviruses (used interchangeably herein with Ad), which cause disease in the respiratory tract, conjunctiva, intestine, urinary tract and liver, have evolved elaborate 25 mechanisms to overcome host antiviral defenses, including at least four of the seven known proteins encoded by the early region 3 (E3) transcription unit which have been reported to inhibit the host immune response to Ad-infected cells (Fejer et al., *J. Virol.* 68:5871-5881, 1994; Sparer et al., *J. Virol.* 70:2431-2439, 1996). One of these proteins is a 19kDa glycoprotein (gp19K), which inhibits CTL-mediated lysis of Ad-infected cells *in vitro* (Efrat 30 et al., *Proc. Natl. Acad. Sci.* 92:6947-6951, 1995). Three other E3 proteins, the 14.7K protein and 10.4K protein in combination with the 14.5K protein (referenced hereinafter as the 10.4K/14.5K complex), protect adenovirus-infected cells against cytolysis and the inflammatory response induced by tumor necrosis factor- α (TNF- α) both *in vitro* and *in vivo* (Sparer et al., *supra*; Krajcsi et al., *J. Virol.* 70:4904-4913, 1996; Dimitrov et al., *J. Virol.* 35 71:2830-2837, 1997). Although the exact stoichiometry of 10.4K and 14.5K proteins in this complex is not known, it is believed to consist of one 14.5K polypeptide in physical

association with a dimer formed by full-length and short forms of the 10.4K polypeptide joined in disulfide linkage. Stewart et al. *supra*.

Efrat et al. have reported that the expression of the one of the Ad E3 genes, i.e. the gene encoding the 19kDa glycoprotein (gp19K), can prolong survival of pancreatic islet allografts. The islets were obtained from transgenic animals prepared to contain the entire E3 genomic DNA from human Ad, however, the gp19K mRNA was prominently expressed with little or no expression of the 10.4K protein which makes up a portion of the 10.4/14.5 complex. The islet allografts survived reportedly due to the expression of the gp19K protein and there was no suggestion in this reference that the 10.4K or 14.5K proteins either separately or in the 10.4K/14.5K complex played any role in the survival of the allografts.

Nevertheless, the 10.4/14.5 complex can protect Ad-infected cells from the inflammatory response in the context of Ad infection (Sparer et al., *supra*) and, although it has not been heretofore recognized, it is possible that the 10.4K/14.5K complex could also provide a novel basis for modulating the immune system in certain disease processes.

Summary of the Invention

In accordance with the present invention, the inventor herein has succeeded in discovering that the Ad 10.4K/14.5K complex inhibits apoptosis mediated by death receptors, in particular Fas or TNFR-1, by removing the death receptor from the cell surface. The present invention, thus, provides a method for inhibiting apoptosis of a cell comprising treating the cell with an effective amount of a 10.4K/14.5K complex referenced herein as RID (Receptor Internalization and Death) or as RID complex. The RID complex reduces the number of molecules of one or more death receptors on the surface of the cell. This down-regulation of the death receptor results from internalization of the receptor to endosomes and degradation of the internalized death receptor by lysozymes. The RID complex is obtained from or derived from the RID α and RID β proteins encoded by the Ad E3 region DNA. Other E3 region-encoded proteins, including the gp19K and 14.7K proteins, are not required to remove the death receptor from the cell surface or to induce apoptosis. Due to the similar structure of TNFR death receptors, and in the common pathway by which they mediate apoptosis, it is believed that RID can inhibit apoptosis mediated by all death receptor members of the TNFR family by promoting their removal from the cell surface.

In one embodiment of the present invention, the cell is treated with RID by administering to the cell a polynucleotide encoding the RID complex, through which the RID complex is expressed in the cell. Alternatively, the treating step comprises administering the RID complex to the cell, preferably in a carrier that facilitates delivery of the complex into the cell. The method can be used to inhibit apoptosis of cells expressing one or more death

receptors of the TNFR family, including but not limited to Fas, TNFR-1, DR3, TRAIL-R1 and TRAIL-R2. Where the cell comprises a tissue, the method is useful for promoting survival of a tissue transplant in a patient or in promoting survival of a tissue under attack in a patient suffering from a degenerative disease, an immunodeficiency disease, an autoimmune disorder or other diseases associated with disregulation of apoptosis mediated by the TNFR death receptors. The method is also useful in inhibiting apoptosis of leukocytes mediated by tumor cells in cancer patients, thereby promoting leukocyte destruction of the patient's tumor cells.

Accordingly, in another embodiment, the present invention provides a method for
10 decreasing apoptosis of target cells in a patient comprising treating the patient with an
effective amount of a RID complex. The target cells express a death receptor which is
downregulated when RID enters the cells.

In yet another embodiment, the invention provides a method for inhibiting leukocyte apoptosis in a patient comprising withdrawing leukocytes from the patient, treating the leukocytes with an effective amount of a RID complex, and administering the treated leukocytes to the patient.

In another embodiment, the present invention provides a composition comprising a RID complex in a carrier suitable for facilitating entry of the RID complex into a cell. As illustrated in Figure 3, a RID complex comprises at least three polypeptides: a full-length Ad E3 10.4K protein having two transmembrane domains (RID α -L), a short form of the 10.4K protein with only one transmembrane domain (RID α -S), and a 14.5K protein (RID β). RID compositions intended for treating humans preferably contain a pharmaceutically acceptable carrier. In one embodiment, the carrier component of the composition comprises a liposome.

The present invention also provides an Ad vector for expressing a RID complex in a cell and to cells transfected with this vector. The vector comprises a nucleotide sequence encoding the RID α and RID β polypeptide components of the complex operably linked to a promoter capable of directing expression of the nucleotide sequence in the cell. A preferred vector consists of 231-10 (SEQ ID NO:2), which expresses functional polypeptides for all of the E3 genes other than *adp*.

30 Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of compositions and methods for inhibiting apoptosis of a cell expressing a death receptor; the provision of compositions and methods for promoting tissue transplant survival in patients; the provision of compositions and methods for treating patients suffering from an autoimmune disease and other disorders associated with

dysfunction of apoptosis regulation; and the provision of compositions and methods for promoting tumor destruction in cancer patients.

Brief Description of the Drawings

5 Figure 1 shows an alignment of the amino acid sequences of the death domains of the death receptor subfamily of TNFR proteins, with residues identical in more than 30% of sequences shaded black and residues conserved in more than 30% of sequences shaded in gray;

10 Figure 2 is a schematic representation of apoptosis mediated by death domain-containing members of the TNF receptor superfamily, with the death receptors Fas, TNFR1, TRAIL-R1, TRAIL-R2 and DR3 depicted by the bars on the extreme right and left sides of the figure, the ligands for these receptors indicated in parenthesis, and showing the association of the death receptors with intracellular proteins in the apoptotic singling cascade at the bottom of the figure;

15 Figure 3 is a schematic representation of a preferred RID complex showing one mature 14.5K polypeptide having an O-glycosylated residue in the extracellular (or luminal) domain and an O-phosphorylated residue in the cytoplasmic domain, and two covalently-linked 10.4K polypeptides, one of which is an uncleaved, full-length form of 10.4K (10.4K-L) having two membrane-spanning regions (diagonal stripes) and the other a cleaved, short form of 10.4K (10.4K-S) with only one transmembrane region;

20 Figure 4 illustrates the amino acid sequences and various domains of preferred embodiments of the RID α and RID β polypeptides, showing in Fig. 4A-4B the long and short forms of the E3 10.4K polypeptides (RID α -L and RID α -S) from Ad serotype 2, Fig. 4C the pre-14.5K (RID β) polypeptide of Ad serotype 5, and in Fig. 4D the mature 14.5K (RID β) polypeptide of Ad serotype 5, with the signal sequences and transmembrane domains underlined and the asterisks indicating sites for disulfide linkage in RID α or for O-phosphorylation in RID β ;

25 Figure 5 is a schematic representation of a model for RID-induced internalization and degradation of Fas and TNFR1 death receptors, showing RID and the death receptor in the plasma membrane, entry of RID and the death receptor into endosomes, transport of these endosomes to lysosomes where the death receptor is degraded, and recycling of RID in endosomes to the cell surface, where it can internalize another death receptor molecule;

30 Figure 6 shows photographs of MCF7-Fas cells (Figs. 6A and 6B) infected with rec700 Ad ("wild-type") or (Figs. 6C and 6D) transiently transfected with pMT2-RID α plus pMT2-RID β which were then treated with an agonist monoclonal antibody to Fas and double-

stained for the adenovirus-encoded DNA binding protein (anti-ADP) (Fig. 6A) and for DNA 4, 6-diamidino-2-phenylindole (DAPI) (Fig. 6B) or double-stained for RID β (Fig. 6C) and DNA (Fig. 6D), with the photographs taken using a 100X Plan apo objective lens;

Figure 7 shows flow cytometry tracings of MCF7-Fas cells which were mock-infected (Fig. 7A) or infected with wild-type Ad (Ad5 and *rec*700) (Figs. 7B-7C) or with the indicated Ad E3 mutant (Figs. 7D-7H) and then incubated with antibodies to Fas (bold trace), transferrin receptor (dashed trace), or control IgG (light trace);

Figure 8 shows flow cytometry tracings of A549 cells which were mock-infected (Fig. 8B) or infected with wild-type Ad (*rec*700) (Fig. 8C) or with the indicated Ad E3 mutant (Figs. 7D-7H) and then incubated with antibodies to Fas (red trace), transferrin receptor (blue trace), or control IgG (black trace), with the cell pattern for mock-infected cells shown in Fig. 8A and R1 indicating the cells that were gated for the analysis;

Figure 9 shows photographs of mock-infected MCF7 cells (Fig. 9A) or MCF7-Fas cells mock-infected (Fig. 9B) or infected with the indicated viruses (Figs. 9C-9H) and then analyzed for Fas by immunofluorescence, with the speckled pattern in Figs. 9C, 9G, and 9H representing putative endosomes and lysosomes containing Fas;

Figure 10 shows an immunoblot of proteins extracted from MCF-7 Fas cells following mock-infection or infection with the indicated wild-type and mutant Ads and stained for Fas (Fig. 10A), transferrin receptor (Fig. 10B) or Ad E1A (Fig. 10C), with molecular weight markers indicated on the right;

Figure 11 shows photographs of COS7 cells transfected with expression plasmids for Fas and RID α (Fig. 11A, 11B), Fas and RID β (Fig. 11C, 11D), or Fas, RID α , and RID β (Fig. 11E-11H) and double-stained for RID α and Fas (Fig. 11A, 11B, 11E, 11F) or for RID β and Fas (Fig. 11C, 11D, 11G, 11H) with arrow in Figs. 11G and H indicate vesicles that appear to contain both RID β and Fas;

Figure 12 shows photographs of *rec*700-infected A549 cells double-stained for Fas and a lysosomal protein, LAMP1 and examined by confocal microscopy, with Fig. 12A showing cells labeled with rabbit anti-Fas antibody and fluorescein isothiocyanate (FITC), Fig. 12B showing cells labeled with mouse anti-LAMP-1 antibody and rhodamine isothiocyanate (RITC), Fig. 12C showing the combined images of Fig. 12A and 12 B, and Fig. 12D showing a perpendicular view of the image in Fig. 12C (arrows), 1 μ m thick, where green indicates Fas, red indicates LAMP-1 and yellow indicates colocalization of Fas and LAMP1 and the bar indicating a distance of 10 μ m;

Figures 13A-13C show photographs of immunofluorescence labeling of Fas in *rec700*-infected cells treated (Fig. 13A) or not treated (Fig. 13B) with baflomycin A1 (Baf), or in *dl309* (RID⁻)-infected cells treated with Baf (Fig. 13C);

Figure 13D shows an immunoblot of proteins extracted from mock-, *rec700*- or 5 *dl309*-infected cells treated (+) or not treated (-) with baflomycin A1 (Baf) and stained for Fas, ERp72, or Ad protein E1B-19K;

Figure 13E shows the immunoblot of Fig. 13D following removal of antibody and restaining for transferrin receptor (TfR);

Figure 14 shows an immunoblot of proteins extracted from COS7 cells transfected 10 with various combinations of plasmids expressing Fas, Shp-1, RID α or RID β as indicated by the "-" and "+" signs and stained for Fas, Erp72 or Shp-1 using appropriate antisera, with the arrows indicating two groupings of bands which correspond to differently glycosylated species of Fas;

Figure 15 shows an immunoblot of proteins extracted from COS7 cells transfected 15 with various combinations of plasmids expressing Fas, chloramphenical acetyl-transferase (CAT), RID α or RID β as indicated by the "-" and "+" signs and stained for Fas, Erp72 or CAT using appropriate antisera, with the arrows indicating two groupings of bands which correspond to differently glycosylated species of Fas;

Figures 16A and 16B are graphs of the amount of lysis of mock-, *rec700*- or *dl7001*-infected Fas-positive mouse P815 cells by activated cytotoxic lymphocytes (CTL) from 20 perforin (-/-) mice (Fig. 16A) or matched perforin (+/+) mice (Fig. 16B) at effector lymphocyte:target ratios of 60:1 (black bars), 20:1 (stippled bars), or 6:1 (open bars);

Figure 16C shows flow cytometry tracings of P815 cells infected with *rec700* (middle plot) or *dl7000* (right dark plot) and then stained for Fas, with the left plot showing the IgG control; 25

Figure 17 is a graph of the amount of lysis of mock- or Ad-infected Fas-positive human A549 cells by natural killer (NK) cells at NK:A549 cell ratios of 10:1(black bar) and 5:1 (striped bar);

Figure 18 shows flow cytometry tracings of human HeLa cells mock-infected (green trace) or infected with *rec700* (red trace) or *dl712*, a mutant that overexpresses RID and E3-30 14.7K (blue trace) and then stained for TNFR1 (Fig. 18A) or Fas (Fig. 18B), with the percentage of cells that stained positive for TNFR1 or Fas indicated at the bottom;

Figure 19 shows flow cytometry tracings of human HeLa cells mock-infected (black trace) or infected with *rec700* (red trace), *dl753* (light blue trace), *dl764* (dark blue trace), 35 *dl712* (green trace), *dl309* (pink trace) and then stained for TNFR1 (Fig. 19A) or Fas (Fig.

19B), with the genotype of each virus and the percentage of cells that stained positive for TNFR1 or Fas indicated at the bottom;

Figure 20 shows flow cytometry tracings of human HeLa cells mock-infected (black trace) or infected with the 231-10 vector, which expresses only the E3 proteins, and then 5 stained for TNFR1 at 24 hr. p.i. (red trace) or 48 hr. p.i. (blue trace);

Figure 21 shows an immunoblot of TNFR1 extracted from A549 cells mock-infected or infected with *rec*700 in which cell surface proteins were labeled by incubation with biotin at the indicated hour p.i.;

Figure 22 shows an immunoblot of TNFR1 (Fig. 22A) and RID β (Fig. 22B) extracted 10 from A549 cells mock-infected or infected with *rec*700 or the 231-10 vector in which cell surface proteins were labeled by incubation with biotin at the indicated hour p.i.;

Figure 23A shows an immunoblot of TNFR1 extracted from A549 cells mock-infected or infected with the indicated virus in which cell surface proteins were labeled by 15 incubation with biotin at 26 h p.i.;

Figure 23B shows an immunoblot of Ad E1B-19K protein extracted from the same cells used in Fig. 23A;

Figure 24 shows a photograph of exposed skin and muscle of the hind flanks of a female C57BL/6 mouse sacrificed 18 days after the flanks were subcutaneously injected with 20 human cancer A549 cells infected with the 231-10 vector, with A549 tumors appearing as whitish-tan masses on each flank;

Figure 25 shows a closer view of the tumor on the right flank of the mouse in Fig. 24;

Figure 26 shows an immunoblot of proteins extracted from an A549 tumor grown in a mouse such as described in Fig. 24;

Figure 27 is a schematic illustration of the structure of the genome of the Ad 231-10 25 vector, with the black horizontal bar representing the backbone of the Ad5 genome, from which the E1 and E3 regions are deleted, as indicated by the triangles below the black bar, and containing an expression cassette with the CMV promoter controlling the E3 genes inserted into the deleted E1 region, as indicated by the triangle to the left, above the black bar, with the transcription unit oriented from right to left as indicated by the arrowhead and 30 restriction endonuclease cleavage sites flanking the CMV-E3 cassette indicated;

Figure 28 illustrates the nucleotide sequence of the 231-10 genome with the numbering beginning with the first base-pair on the conventional left side of the Ad5 genome as shown in Fig. 27 and proceeding to the last base-pair at the right side of the genome;

Figure 29 shows an immunoblot of E3 RID β , 14.7K, and gp19K proteins expressed in 35 A549 cells infected with the 231-10 vector and detected at the days p.i. indicated, with lane A

containing proteins extracted from 231-10-infected cells at 1 day p.i. following treatment with 1- β -D-arabinofuransylcytosine (araC) at 2 h p.i.; and

Figure 30 shows a photograph of A549 cells infected with the 231-10 vector and gp19K, RID β , and 14.7K proteins detected by indirect immunofluorescence.

5

Detailed Description of the Invention

The present invention is based on the discovery that the Ad RID complex inhibits apoptosis mediated by death receptors, and in particular by Fas and TNFR1. Some of the molecular events involved in apoptosis induced through death receptors of the TNFR family 10 are illustrated in Fig. 2. Fas (bar on the extreme right) is localized on the cell surface. When FasL engages Fas on the outside of the cell (top of Fig. 2), Fas associates with proteins within the cell (bottom of Fig. 2). First, Fas binds a protein named FADD through their corresponding death domains and then the Fas/FADD complex binds the protein named Caspase 8 through another region in FADD and Caspase 8 named the "death effector" domain. This binding activates the enzymatic activity of Caspase 8, an "initiator" caspase. Activated Caspase 8 cleaves other caspases (effector caspases), which then cleave other proteins, and apoptosis ensues. Apoptosis induced through TNFR is very similar, except that an additional protein, named TRADD, is involved. TNF engages TNFR1, causing it to bind TRADD through death domains in TNFR1 and TRADD (left part of Fig. 2). The 15 TNFR1/TRADD complex then binds FADD through their death domains and this is followed by binding to Caspase 8, etc. TRAIL-R1, TRAIL-R2, and DR3 are believed to undergo a similar binding cascade to activate caspases, although the ligand that triggers apoptosis through DR3 is unknown.

RID inhibits apoptosis by means of an internalization and degradation mechanism 25 common to all death receptors. As illustrated in Figure 2, RID shuttles the death receptor from the cell surface to lysosomes where the receptors are degraded. This model is supported in part by the fact that the RID complex has two motifs in its intracellular portion that are known to play a role in the internalization of some cell surface receptors and their transport to lysosomes. These motifs are a dileucine motif (LL), which is present in RID α , and a 30 tyrosine-based motif in RID β , which is YXX ϕ , where Y is tyrosine, X is any amino acid, and ϕ is an aromatic or bulky hydrophobic amino acid such as phenylalanine, tyrosine, tryptophan and proline. It is believed that RID acts through the LL and YXX ϕ motifs to cause Fas or TNFR1 to be internalized into early/sorting endosomes. Again, acting through the LL and YXX ϕ motifs, RID mediates transport of the early endosomes to late endosomes and then to 35 lysosomes where the receptors are degraded. RID then recycles back to the cell surface in

endosomes where it repeats this process. Additional evidence supporting this model is as follows: (1) RID co-localizes with Fas on the cell surface as well as in vesicles; (2) degradation of Fas is inhibited by bafilomycin A1, an inhibitor of late endosome function; (3) the RID proteins are very stable, as indicated by pulse-chase experiments, whereas Fas is very unstable in the presence of RID; and (4) mutation of the LL motif severely reduces the function of RID, and conversion of the Y in the YXX ϕ motif abolishes the function of RID.

Because of their similar structures and common apoptotic pathway, it is believed that all death receptors of the TNFR family can be removed from the cell surface by RID via internalization into endosomes and subsequent degradation in lysosomes. Thus, RID will inhibit apoptosis mediated by any member of the TNFR death receptor family. As such, RID should be useful to promote survival of cells and tissues in the treatment of diseases such as degenerative diseases, immune disorders including autoimmune disorders, ischemic injury such as caused by myocardial infarction, stroke induced neuron death and reperfusion injury, alcohol-induced hepatitis, diseases caused by viral infection, such as AIDS and fulminant hepatitis, and cancer. RID is also useful in promoting survival of tissue transplants in transplant recipients.

Thus, in one embodiment the invention provides a method for inhibiting apoptosis of a cell comprising treating the cell with an effective amount of a Receptor Internalization and Degradation (RID) complex. Cells which can be treated by this method express one or more death receptors of the TNFR family, which includes Fas, TNFR1, DR3, TRAIL-R1, TRAIL-R2 and any subsequently discovered family member characterized by the presence of a death domain. Cells expressing a death receptor can be identified by methods known in the art, such as incubating the cells with one or more death receptor ligands followed by evaluating the cells for apoptosis, detecting death receptor molecules on the cell surface with an antibody against the death receptor, or detecting mRNA molecules that encode the death receptor. Cell death by apoptosis is readily recognizable and includes cytoplasmic and nuclear condensation, loss of membrane integrity and extensive fragmentation of chromosomal DNA, which forms a characteristic ladder when analyzed by gel electrophoresis. Vaux, D., *Proc. Natl. Acad. Sci.* 90:786-789, 1993. Antibodies against the TNFR death receptors are either commercially available or can be readily prepared using standard techniques.

The RID complex used in the method comprises at least one of each of the following polypeptides: a RID α -L polypeptide, a RID α -S polypeptide, and a RID β polypeptide. RID α and RID β are synonymous with the 10.4K and 14.5K proteins, respectively, which are encoded by two genes in the Ad E3 region. The basic structures of these polypeptides in a membrane are illustrated in Fig. 3. RID α -L comprises a first transmembrane domain, which

is an uncleaved signal sequence, an extracellular domain, an internal transmembrane domain, and a cytoplasmic domain. RID α -S lacks the signal sequence and thus comprises the extracellular domain, the internal transmembrane domain and the cytoplasmic domain. RID β comprises an extracellular domain, which preferably lacks the signal sequence as shown in Fig. 4D, a transmembrane domain and a cytoplasmic domain. When the RID complex is localized in membrane structures and vesicles within the cell, the extracellular domain is located in the lumen of these membranes and vesicles.

In preferred embodiments, the RID α -S and RID α -L polypeptides are covalently joined by a disulfide bond between cysteine residues in their extracellular domains which correspond by alignment with the Cys₃₁ residue of the Ad2 10.4K protein (Fig. 4A). Also, RID β preferably has a mucin type O-linked oligosaccharide attached to one or more amino acids in the extracellular domain and/or is phosphorylated at one or two serines in the cytoplasmic domain. (See Krajcsi et al., *Virology* 187:492-498, 1992; Krajcsi et al., *Virology* 188:570-579, 1992.) The location of these residues in RID β polypeptides encoded by E3 genes of different Ad serotypes can be determined by alignment with the amino acid sequence for the 14.5K protein of Ad5, which is shown in Fig. 4C.

A RID complex made by Ad *in vivo* is believed to contain RID α -L, RID- α S and RID β (lacking the signal sequence) polypeptides in about a 1:1:1 ratio. However, it is possible that various ratios of these polypeptides will be functional or that in some cases different ratios will be required to provide a functional complex.

The amino acid sequences of the RID α -L, RID α - β and RID β polypeptides comprising the RID complex may be identical to those of naturally-occurring Ad RID α (10.4K) and RID β (14.5K) proteins from any Ad serotype or may comprise functional variants of such naturally-occurring sequences. As stated above, the genes encoding the RID α and RID β proteins are highly conserved among Ad serotypes. These genes are also conserved in Ads from some non-human species. Thus, it is believed that their encoded products should function very similar to the RID α and RID β polypeptides from Ad2 and Ad5, which were used in the experiments described herein. In addition, the invention includes the use of RID complexes in which the RID α -L, RID α -S, and RID β polypeptides comprise homologous amino acid sequences, i.e., encoded by the same Ad serotype, or that comprises heterologous sequences, i.e., encoded by two or more Ad serotypes. Thus, for example, a RID complex may comprise (1) a RID α -L polypeptide comprising the RID α -L amino acid sequence from Ad2, (2) a RID α -S polypeptide comprising the RID α -S amino acid sequence from Ad5, and (3) a RID β polypeptide comprising the RID β amino acid sequence from Ad9. Preferably, the RID complex comprises polypeptides whose amino acid sequences correspond

to serotypes from the same subgroup. More preferably, the RID complex comprises RID α -S and RID α -L polypeptides encoded by the RID α gene of Ad2 and a RID β polypeptide encoded by the RID β gene of Ad5.

A functional variant of a naturally-occurring RID α or RID β sequence contains one or more amino acid substitutions in that sequence which do not destroy the ability of the resulting polypeptide to function in a RID complex to inhibit apoptosis. Preferably, amino acid substitutions in functional variants are conservative amino acid substitutions, which refer to the interchangeability of residues having similar side chains. Conservatively substituted amino acids can be grouped according to the chemical properties of their side chains. For example, one grouping of amino acids includes those amino acids have neutral and hydrophobic side chains (A, V, L, I, P, W, F, and M); another grouping is those amino acids having neutral and polar side chains (G, S, T, Y, C, N, and Q); another grouping is those amino acids having basic side chains (K, R, and H); another grouping is those amino acids having acidic side chains (D and E); another grouping is those amino acids having aliphatic side chains (G, A, V, L, and I); another grouping is those amino acids having aliphatic-hydroxyl side chains (S and T); another grouping is those amino acids having amine-containing side chains (N, Q, K, R, and H); another grouping is those amino acids having aromatic side chains (F, Y, and W); and another grouping is those amino acids having sulfur-containing side chains (C and M). Preferred conservative amino acid substitutions groups are: R-K; E-D, Y-F, L-M; V-I, and Q-H. In addition, conservative amino acid substitutions as used herein is intended to include substitutions which are present at corresponding positions in sequences from different Ad serotypes.

A functional variant as used herein can also include modified sequences in which one or more amino acids have been inserted, deleted, or replaced with a different amino acid or a modified amino acid or unusual amino acid, as well as modifications such as glycosylation or phosphorylation so long as the polypeptide containing the modified sequence retains the biological activity of a RID α or RID β polypeptide. By retaining the biological activity, it is meant that the modified polypeptide can function to form a RID complex with anti-apoptotic activity.

In one embodiment, the cell is treated with the RID complex by administering to the cell a polynucleotide encoding the RID complex. The polynucleotide comprises a nucleotide sequence encoding a RID α polypeptide and a RID β polypeptide operably linked to a promoter that produces expression of the RID complex in the cell. In one variation of this embodiment, the polynucleotide can contain portions of the Ad E3 region in addition to that portion encoding RID α and RID β . However, the polynucleotide predominantly expresses the

RID α and RID β proteins over any other Ad proteins. Alternatively, actions on cell apoptosis resulting from expression of the polynucleotide are predominantly due to the RID complex rather than any other protein expressed by the polynucleotide. The polynucleotide can comprise an expression plasmid, a retrovirus vector, an Ad vector, an adenovirus associated vector (AAV) or other vector used in the art to deliver genes into cells. Alternatively, the polynucleotide can be administered to the cell by microinjection.

In embodiments where the cell being treated is in a patient, such as cells comprising a tissue transplant or a tissue involved in an autoimmune disorder, the polynucleotide encoding RID is administered to the patient. Any of the vectors discussed above can be used. It is also contemplated that the RID complex be administered by coinfection with a replication-defective Ad expressing RID and another replication competent Ad that complements the replication defective virus to increase the expression of RID in the infected cells.

Preferably, the polynucleotide is selectively delivered to target cells within the patient so as not to affect apoptosis in other tissues. Targeted delivery of the polynucleotide can be done for example by using delivery vehicles such as polycations, liposomes or viral vectors containing targeting moieties that recognizes and binds a specific marker on the target cell. Such methods are known in the art, see, e.g., U.S. Patent No. 5,635,383. Another targeted delivery approach uses viral vectors that can only replicate in specific cell types which is accomplished by placing the viral genes necessary for replication under the transcriptional control of a response element for a transcription factor that is only active in the target cell. See, e.g., U.S. Patent No. 5,698,443.

In other embodiments of the invention, the cell is treated by administering to the cell a composition comprising a RID complex. The RID complex for use in such embodiments can be prepared by a variety of means. For example, the RID complex can be isolated from the membranes of Ad-infected cells or cells transfected with a nucleotide sequence encoding the RID α and RID β polypeptides. Alternatively, the polypeptide components of the complex can be expressed in separate cell cultures, extracted into an appropriate buffer and mixed *in vitro*. RID α and RID β polypeptides can also be chemically synthesized and mixed to form the complex. The RID complex can then be tested for the ability to inhibit apoptosis of a cell expressing a death receptor as described herein for Fas and TNFR1.

Preferably, the RID complex is administered with a carrier that facilitates delivery of the RID complex into the cell, such as liposomes. Where the RID complex is being administered to a patient, the liposomes can have targeting moieties exposed on the surface such as antibodies, ligands or receptors to specific cell surface molecules to limit delivery of RID to targeted cells. Liposome drug delivery is known in the art (see, e.g., Amsalem et al.,

Chem. Phys. Lipid 64:219-237, 1993). Alternatively, one or more of the polypeptides of the complex can be modified to include a specific transit peptide that is capable of delivering the peptide into the cytoplasm of a cell or the complex can be delivered directly into a cell by microinjection.

5 Compositions comprising a RID complex can be administered by any suitable route known in the art including, for example, intravenous, subcutaneous, intramuscular, transdermal, intrathecal or intracerebral or administration to cells in *ex vivo* treatment protocols. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow release formulation. For treating tissues in the central nervous system, administration can be by injection or infusion into the cerebrospinal fluid (CSF). When it is intended that the RID complex be administered to cells in the central nervous system, administration can be with one or more agents capable of promoting penetration of the protein complex across the blood-brain barrier.

10 The RID complex can also be linked or conjugated with agents that provide desirable pharmaceutical or pharmacodynamic properties, including for example, substances known in the art to promote penetration or transport across the blood-brain barrier such as an antibody to the transferrin receptor (Friden et al., *Science* 259:373-377, 1993), a polymer such as polyethylene glycol to obtain desirable properties of solubility, stability, half-life and other pharmaceutically advantageous properties Davis et al. *Enzyme Eng* 4:169-73, 1978; Burnham, 15 *Am J Hosp Pharm* 51:210-218, 1994).

20 For nonparental administration, the compositions can also include absorption enhancers which increase the pore size of the mucosal membrane. Such absorption enhancers include sodium deoxycholate, sodium glycocholate, dimethyl- β -cyclodextrin, lauroyl-1-lysophosphatidylcholine and other substances having structural similarities to the 25 phospholipid domains of the mucosal membrane.

The compositions are usually employed in the form of pharmaceutical preparations. Such preparations are made in a manner well known in the pharmaceutical art. One preferred preparation utilizes a vehicle of physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers such as physiological concentrations of other non-toxic salts, five percent aqueous glucose solution, sterile water or the like may also be used. It may also be desirable that a suitable buffer be present in the composition. Such solutions can, if desired, be lyophilized and stored in a sterile ampoule ready for reconstitution by the addition of sterile water for ready injection. The primary solvent can be aqueous or alternatively non-aqueous.

30 35 The carrier can also contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate

of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmaceutically-acceptable excipients for modifying or maintaining release or absorption or penetration across the blood-brain barrier. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dosage 5 or multi-dose form or for direct infusion by continuous or periodic infusion.

It is also contemplated that certain formulations comprising the RID complex are to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium 10 phosphate, alginates, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving 15 agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic degradation and/or substances which promote absorption such as, for example, surface active agents.

The RID complex is administered to patients in an amount effective to inhibit 20 apoptosis of target cells within the patient. The specific dose is calculated according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also be calculated dependent upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those of ordinary skill in the art. Such 25 calculations can be made without undue experimentation by one skilled in the art in light of the activity disclosed herein in cell death assays. Exact dosages are determined in conjunction with standard dose-response studies. It will be understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of 30 composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration. Dose administration can be repeated depending upon the pharmacokinetic parameters of the dosage formulation and the route of administration used.

The compositions and methods of the invention are contemplated for use in 35 promoting survival of tissue transplants. For example, the tissue can be treated *in vitro* with the RID complex and the treated tissue then introduced into the transplant. In addition,

previously transplanted tissues can be treated with RID by administering the RID complex to the transplant recipient. In either scenario, it is contemplated that the RID complex can be administered as a protein formulation or as a polynucleotide expressing the complex.

In another embodiment, the RID complex is used to promote the survival of leukocytes in cancer patients. The leukocytes can be treated *in vivo* by administering to the patient a polynucleotide expressing RID or a composition containing the RID complex. Preferably, the polynucleotide or RID complex is targeted to the leukocytes by one of the targeting methods discussed above. For example, cytotoxic T cells could be targeted by using an antibody against the CD8 marker and natural killer cells targeted by use of an antibody against the CD16 marker. Alternatively, the leukocytes can be removed from the patient, treated with the RID complex *ex vivo*, and the treated leukocytes then returned to the patient.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

Example 1

This example illustrates inhibition of Fas-mediated apoptosis by adenovirus E1B and E3 proteins.

Human breast adenocarcinoma cells expressing Fas (MCF7-Fas) (Jäättela et al., *Oncogene* 10:2297-2305, 1995) were infected with *rec700* or with an adenovirus mutant lacking expression of one or more of the RID α , RID β , E3-14.7K and E1B-19K proteins. *rec700* is an Ad5-Ad2-Ad5 "wild-type" recombinant whose genome consists of the Ad5 EcoRI A (map positions 0 to 76), Ad2 EcoRI D (map positions 76 to 83), and Ad5 EcoRI B (map positions 83 to 100) fragments (Wold et al., *Virol.* 148:168-188, 1986). *rec700* is the parental virus of E3 mutants with 700 or 7000 numbers. The infected cells were treated with a monoclonal antibody to Fas, CH-11, which acts as an agonist of Fas and induces apoptosis. The cells were then fixed and stained for DNA and for the adenovirus DNA binding protein (DBP). Experimental details are provided in the footnote to Table 1.

Examples of apoptotic and non-apoptotic nuclei in *rec700*-infected cells are shown in Figs. 6A and 6B. Most cells were infected as indicated by the speckled staining of DBP in the nucleus (Fig. 6A), and these nuclei were non-apoptotic (Fig. 6B). Two uninfected cells were apoptotic (arrows in Figs. 6A and 6B) as evidenced by the presence of shrunken and irregular nuclei with condensed DNA that often fluoresced very brightly above the plane of

focus for non-apoptotic nuclei. The percentage of apoptotic and non-apoptotic nuclei was scored in *rec700*- or mutant-infected cells staining for DBP and the quantitative results are shown in Table 1 below.

5 Table 1. Fas Agonist-induced Apoptosis in MCF7-Fas Cells Infected with Ad Mutants¹

Virus Mutant	Ad DNA Binding Protein-Positive Cells ²	
	Apoptotic	Non-apoptotic
<i>rec700</i> (wild type)	0.1 ³	99.9 ³
<i>pm760</i> (E1B-19K ⁺ , RID ⁻)	0.7	99.3
<i>dl309</i> (E1B-19K ⁺ , RID ⁻)	0.1	99.9
<i>dl748</i> (E1B-19K ⁺ , RID ⁻)	0.6	99.4
<i>dl764</i> (E1B-19K ⁺ , RID ⁻)	0.2	99.8
<i>lp5</i> (E1B-19K ⁺ , RID ⁻)	9.9	90.2
<i>dl250</i> (E1B-19K ⁺ , RID ⁻)	10.4	89.6
<i>dl111</i> (E1B-19K ⁺ , RID ⁻)	87.2	12.8
<i>dl118</i> (E1B-19K ⁺ , RID ⁻)	94.1	5.9

¹MCF7-Fas cells were infected with 250 PFU per cell of virus except for *lp5*, *dl250*, *dl111*, and *dl118* where 10 PFU per cell was used. At 21 h post-infection (p.i.), cells were treated for 10 22 h with the CH-11 agonist mAb to Fas (200 ng/ml) (Panvera, Madison, WI) plus cycloheximide (25 µg/ml). Cells were fixed and stained for the Ad DNA binding protein (DBP) using a rabbit antiserum (obtained from Maurice Green, St. Louis University) and goat anti-rabbit IgG (fluorescein conjugate) and for DNA using 4, 6-diamidino-2-phenylindole (DAPI). Typical apoptotic and non-apoptotic nuclei are shown in Fig. 6B, which is from the same experiment. Nuclei of *dl111*- or *dl118*-infected cells not treated with Fas agonist were not apoptotic (not shown), indicating that the apoptosis observed was not due to the *cyc deg* phenotype of E1B-19K-negative mutants (Subramanian et al., *J. Virol.* 52:336-343, 1984).

20 ²At least 1000 DBP-positive cells were counted per sample.

³Percent of apoptotic and non-apoptotic nuclei in cells staining for DBP.

In cells infected with *rec700* or mutant *pm760*, which expresses both E1B-19K and RID, very few nuclei were apoptotic. Cells infected with mutants expressing E1B-19K but lacking RID α and E3-14.7K (*dl748*), or lacking RID β (*dl764*), or lacking each of RID α , RID β , and E3-14.7K (*dl309*) also had very few apoptotic nuclei. However, only about 10% of cells infected with *lp5* and *dl250*, which lack E1B-19K but express RID, had apoptotic nuclei, while about 90% of the nuclei were apoptotic in cells infected with *dl111* and *dl118*, which lack expression of RID α , RID β , E3 14.7 K and E1B-19K. These results indicate that adenovirus has two proteins that independently inhibit Fas-induced apoptosis, RID and/or E3-14.7K in the E3 transcription unit and E1B-19K in the E1B transcription unit. This result observed with E1B-19K is consistent with an earlier report (Hashimoto, S., et al., *Int. Immunol.* 3:343-351, 1991). Data below show that RID inhibits Fas-induced apoptosis.

Example 2

This example illustrates that the RID complex is sufficient to inhibit apoptosis.

To address whether RID is sufficient to inhibit Fas-induced apoptosis, plasmids expressing RID α or RID β from the Ad major late promoter plus SV40 enhancer were prepared by cloning the gene for RID α or RID β into the pMT2 vector (Mazzarella, R. A. & Green, M. J. *Biol. Chem.* 262: 8875-8883, 1987) to generate pMT2-RID α and pMT2-RID β . MCF7-Fas cells were transiently transfected with pMT2-RID α plus pMT2-RID β , pMT2-RID β alone, or pMT2 alone (2.5 μ g for each plasmid). After 38 h, cells were treated for 9 h with the CH-11 agonist mAb to Fas (500 ng/ml) plus cycloheximide (25 μ g/ml), fixed in methanol with DAPI, and stained for RID β using the rabbit P118-132 antipeptide antiserum (Tollefson et al., *Virology* 175:19-29, 1990).

Examples of apoptotic and non-apoptotic nuclei in the cells co-transfected with pMT2-RID α and pMT2-RID β are shown in Figs. 6C and 6D. The cell transfected with RID α plus RID β (arrow in Fig. 6C) was non apoptotic (arrow in Fig. 6D). RID β -negative cells usually had apoptotic nuclei (most cells in Fig. 6D). Of 2000 cells counted in random fields, 173 RID β -positive cells were seen, and only 26% of these had apoptotic nuclei. In the transfection with RID β alone, and with 2000 cells counted, 101 RID β -positive cells were seen, 80% of which had apoptotic nuclei. With pMT2 alone, 62% of the total nuclei were apoptotic. These results indicate that RID (i.e. RID α plus RID β), but not RID β alone, is sufficient to inhibit Fas-induced apoptosis.

Example 3

This example illustrates that RID down-regulates Fas from the cell-surface of adenovirus-infected human breast carcinoma cells.

To investigate how RID inhibits apoptosis, MCF7-Fas cells were infected with 5 adenovirus serotype 5 (Ad5), *rec700*, or an Ad mutant lacking expression of one or more of RID α , RID β , and E3-14.7K proteins. At 28 h p.i., cells were detached using 0.025% EDTA, then resuspended in FACS buffer (1X PBS, 2% FBS). Approximately 1×10^6 cells were pelleted and resuspended in 50 μ l FACS buffer containing antibodies against human Fas (UB2 IgG mAb) (Panvera) (10 μ g/ml), the human transferrin receptor 10 (Boehringer/Mannheim, Indianapolis, IN) (2.5 μ g/ml) and purified mouse IgGy (PharMingen, San Diego, CA) (5 μ g/ml) as an iso-type control. In common with Fas, the transferrin receptor is a cell surface receptor. Cells were incubated with the primary antibodies, washed with cold FACS buffer, incubated with 20 μ g/ml of goat anti-mouse FITC-conjugated antibody (ICN), washed, then analyzed on a FACScaliber flow cytometer (Becton Dickinson, 15 Mountain View, CA). The data were analyzed with Cell Quest software (Becton Dickinson) and are shown in Figure 7.

Nearly all Fas (bold trace in Fig. 7) was cleared from cells infected with Ad5 or *rec700* (Figs. 7B, 7C). Transferrin receptor (dashed trace) was not affected. Fas was not cleared from cells infected with mutants lacking RID α and/or RID β , namely *d1309* (lacks RID α , RID β , E3-14.7K) (Fig. 7D), *d1748* (lacks RID α) (Fig. 7E), and *d1764* (lacks RID β) 20 (Fig. 7F). Fas was down-regulated by *d1758* (RID-positive, lacks E3-14.7K) (Fig. 7G) and *pm760* (overexpresses RID α and RID β) (Fig. 7H). These results indicate that RID (i.e. RID α and RID β) is necessary to clear Fas from the surface of Ad-infected MCF7-Fas cells. Other Ad proteins, including E3-14.7K and E1B-19K, are not required.

25

Example 4

This example illustrates that RID down-regulates Fas from the cell-surface of adenovirus-infected human lung adenocarcinoma cells.

To determine if RID can remove Fas from the surface of other cell types, the human 30 A549 cell line was examined. A549 cells are derived from a human lung adenocarcinoma. A549 cells were mock-infected or infected with *rec700*. At 26 h p.i., cells were suspended in FACS buffer containing mouse IgGy, anti-human-Fas UB2 IgG monoclonal antibody (Panvera), or antibody against the human transferrin receptor (Boehringer/Mannheim), incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody, and

analyzed on a FACScaliber flow cytometer using Cell Quest software (Becton Dickinson). The results are shown in Fig. 8.

With mock-infected cells (Fig. 8B), there was strong staining for both Fas (the red trace in Fig. 8) and transferrin receptor (the blue trace in Fig. 8). With *rec700* or *pm760*, a virus mutant that overexpresses RID (i.e., RID α plus RID β) and underexpresses other Ad E3 proteins, Fas was completely cleared from the cell surface whereas the transferrin receptor was not affected (Figs. 8C, 8H). With three virus mutants that lack both RID α and RID β (*dl309*), RID β only (*dl764*), or RID α (*dl748*), Fas was not cleared from the cell surface (Fig. 8, Panels E, F, and G). With *dl758*, a mutant that lacks only E3-14.7K and that expresses RID α and RID β , Fas was down-regulated to the same extent as with *rec700* and *pm760*. Therefore, the E3-14.7K protein is not required to down-regulate cell surface Fas. Recently, RID was reported to clear Fas from the cell surface in two other human cell lines, HT-29.14S and ME-180 (Shisler et al., *J. Virol.* 71:8299-8306, 1997). These results have been confirmed with HT-29.14S and ME-180 cells (data not shown). Thus, RID stimulates the removal of Fas from the cell surface of at least four different cell types, MCF7-Fas, A549, HT-29.14S, and ME-180 cells.

Example 5

This example illustrates that Fas molecules removed from the cell surface by RID are internalized into vesicles and then degraded in lysosomes.

Many receptors are internalized into endosomes. Accordingly, MCF7-Fas cells were mock-infected or infected with *rec700* or with an E3 Ad mutant. MCF7 cells were mock-infected as a control. At 19 h p.i., cells were fixed in methanol and stained for Fas using the ZB4 mAb (Panvera) and goat anti-mouse IgG (Texas red conjugate). The results are shown in Figure 9.

Fas was not detected in mock-infected parental MCF7 cells (Fig. 9A), but was readily apparent on the surface of MCF7-Fas cells (Fig. 9B). In cells infected with *rec700*, Fas was in numerous vesicles and there was no cell surface staining (Fig. 9C). These vesicles are likely to be endosomes and lysosomes containing Fas. These vesicles were not observed with *dl309*, *dl748*, or *dl764* (lack RID α and/or RID β), whereas in each case, strong Fas staining was apparent at the plasma membrane (Figs 9D-9F). Vesicles staining for Fas were seen with *dl758* and *pm760*, both of which express RID (Figs. 9G, 9H).

Some receptor types internalized into endosomes are targeted to lysosomes where they are degraded. To determine whether Fas was degraded in Ad-infected cells expressing RID, MCF7-Fas cells were mock-infected or infected with wild-type Ad or an E3 mutant

lacking expression of one or more of RID α , RID β , and 14.7K proteins, then at 27 h p.i. proteins were extracted, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto an Immobilon-P membrane. After blocking, membranes were incubated with rabbit anti-Fas antiserum (Santa Cruz Biotechnology, Inc., 5 Santa Cruz, CA), mouse anti-transferrin receptor mAb OKT9 (ATCC), or mouse anti-E1A mAb M73. Membranes were incubated with the appropriate peroxidase-conjugated secondary antibody (ICN). Proteins were detected with ECL reagents (Amersham Life Sciences, Arlington Heights, IL) and the results are shown in Fig. 10.

Fas was degraded in cells infected with viruses that express both RID α and RID β 10 (Ad5, Ad2, rec700, dl758, pm760) (Fig. 10A). Transferrin receptor was not degraded in these same extracts (Fig. 10B). Fas expression was actually stimulated in cells infected with mutants that lack RID α and/or RID β (Fig. 10A, compare Mock with dl309, dl748, and dl764). The Ad-coded E1A proteins were expressed at similar levels (Fig. 10C), indicating 15 that all infections were equivalent. These and the above results establish that RID (i.e. RID α and RID β) functions in the internalization of Fas into putative endosomes, the degradation of Fas, and the inhibition of Fas-induced apoptosis.

RID has been reported to stimulate the internalization of EGFR into vesicles and its degradation in lysosomes (Carlin et al., *Cell* 57:135-144, 1989; Tollefson et al., *J. Virol.* 65:3095-3105, 1991). When the epidermal growth factor receptor (EGFR) interacts with its 20 ligand, EGF, EGFR is internalized into early endosomes which are transported to late endosomes which fuse with lysosomes, where EGFR is degraded. This process results in attenuation of signal transduction through EGFR. Many receptors are degraded by the endosome-lysosome pathway in response to ligand. To determine if RID-induced degradation of Fas is occurring through this pathway, the following experiments were performed.

The first experiment, which was described in the copending provisional application, 25 examined Fas localization in COS cells transiently co-transfected with combinations of expression plasmids for Fas, RID α and RID β . The following plasmids were used, the pMT2-RID α and pMT2-RID β plasmid vectors described in Example 2, and pcDNA3-Fas, which expresses Fas from the human cytomegalovirus promoter (CMV). C0S7 cells were 30 transfected (Mazzarella, R. A. & Green, M. J. *Biol. Chem.* 262:8875-8883, 1987) with 1 μ g each of pMT2-RID α plus pcDNA3-Fas, pMT2-RID β plus pcDNA3-Fas, or pMT2-RID α , pMT2-RID β , and pcDNA3-Fas. After 30 h, cells were fixed in methanol with DAPI and stained for Fas using the ZB4 mAb, for RID α using the rabbit P77-91 antipeptide antiserum, or for RID β using the rabbit P118-132 antipeptide antiserum (Tollefson et al., *J. Virol.*

64:794-801, 1990; Tollefson et al., *Virology* 175:19-29, 1990). The results are shown in Figure 11.

With cells co-transfected with expression plasmids for RID α plus Fas, or RID β plus Fas, Fas was localized on the cell surface (Fig. 11B, 11D). In contrast, with cells triple-transfected with expression plasmids for RID α , RID β , and Fas, Fas was in vesicles rather than the cell surface (Fig. 11F, 11H). RID β staining was typical of the endoplasmic reticulum (ER) and plasma membrane, a probable site of RID action (Stewart et al., *J. Virol.* 69:172-181, 1995); many vesicles containing RID β appeared to co-localize with vesicles containing Fas (arrows in Fig. 11G and 11H). Distribution to the ER was also characteristic of RID α (Fig. 11E), and in some cells the plasma membrane was stained (not shown). RID α did not co-localize with Fas-containing vesicles. Thus, RID (i.e. RID α plus RID β) is sufficient to internalize Fas into vesicles.

In a second experiment, Fas localization was examined in Ad-infected cells. Human A549 cells were infected with rec700 fixed using 3.7% paraformaldehyde followed by methanol/DAPI (4,6-diamidino-2-phenylindole). Cells were double-stained for Fas and LAMP1, which is a lysosomal protein (Carlsson et al., *J. Biol. Chem.* 15:18911-18919, 1988), using a rabbit anti-Fas antibody (Santa Cruz Biotechnology) and the BB6 mouse anti-human-LAMP-1 monoclonal antibody (Carlsson et al., supra), followed by goat anti-rabbit IgG-FITC and goat anti-mouse IgG-RITC (rhodamine isothiocyanate) (Cappel ICN). Cells were examined using a Zeiss LSM 410 scanning laser confocal microscope with LSM 410 software. The results are shown in Figure 12.

Green, red, and yellow vesicles contain Fas (Fig. 12A), LAMP1 (Fig. 12B), or both Fas and LAMP1 (Fig. 12C, 12D), respectively. The many yellow vesicles establish that Fas co-localizes with LAMP1 in lysosomes. The Fas-containing green vesicles may be endosomes. Similar results were obtained with another lysosomal protein, CD63 (data not shown).

To obtain additional evidence supporting the involvement of the endosome-lysosome pathway in RID-induced Fas degradation in Ad-infected cells, the effect of Bafilomycin A1 (Baf) treatment was investigated. Baf specifically inhibits the vacuolar-type H⁺-ATPase, preventing vesicle acidification and trafficking of receptors from late endosomes to lysosomes (Yoshimori et al., *J. Biol. Chem.* 266:17707-17712, 1991; van Weert et al., *J. Cell. Biol.* 130:821-834, 1995). A549 cells were mock-infected or infected with rec700 or d309 (lacks RID). At 13 h after infection, cells were treated with Baf (0.1 μ M) for 12 h and then immunostained for Fas. In a separate experiment, cells were treated with Baf at 6 h after

infection and processed for immunoblot analysis 18 h later. The results are shown in Figure 13.

When wild-type Ad-infected cells were treated with Baf, Fas was cleared from the cell surface but it accumulated in vesicles (Fig. 13A) rather than being degraded as in untreated cells (Fig. 13B). Baf did not affect cell surface Fas in cells infected with a mutant lacking RID (*d1309*) (Fig. 13C). Immunoblot analysis of proteins extracted from these cells indicated that Baf blocked the degradation of Fas in wild-type Ad-infected cells (Fig. 13D). Baf did not affect the abundance of Fas in mock-infected cells or in cells infected with the RID-minus mutant. Neither virus infection nor Baf affected the abundance of Erp72 (Fig. 13D), a cellular protein localized in the endoplasmic reticulum (Mazzarella et al., 1990). Also, neither virus infection nor Baf significantly affected the level of another cellular protein, the transferrin receptor (Fig. 13E). The infections were equivalent as indicated by the E1B-19K levels of the Ad-encoded protein (Fig. 13D). These confocal microscopy and Baf data provide strong evidence that RID causes Fas to be degraded in lysosomes in Ad-infected cells.

Example 6

This example illustrates that the RID proteins are sufficient to promote the degradation of Fas.

COS cells were transiently transfected with different combinations of pMT2-RID α , MT2-RID β , pcDNA3-Fas, and pBUC-Shp-1, which expresses a mammalian cell protein named Shp-1. At 36 h post-transfection, cells were treated with cycloheximide (25 μ g/ml) for 12 h and at 48 h post-transfection, proteins were extracted and analyzed for Fas, Shp-1, or ERp72 by immunoblot using rabbit antisera to Fas (Santa Cruz), Erp72 (Mazzarella et al., 1990), or Shp-1 (Plas et al., 1996) (Tollefson et al., Nature 392:726-730 (1998)). The results are shown in Fig. 14.

In cells transfected with pcDNA3-Fas and/or pBUC-Shp-1, expression of Fas and/or Shp-1 proteins was readily detected by immunoblot (Fig. 14, lanes b-d). For Fas, two groupings of bands were detected (indicated by the arrows), which represent differentially glycosylated species of Fas. The anti-Fas antibody also reacted with an unknown cellular protein that migrated between the two sets of Fas protein bands. When pMT2-RID α or pMT2-RID β were co-transfected with pcDNA3-Fas and pBUC-Shp-1, there was a marginal decrease in Fas and Shp-1 (Fig. 14, lanes e and f). However, when both pMT2-RID α and pMT2-RID β were co-transfected with pcDNA3-Fas and pBUC-Shp-1, the Fas bands were reduced to nearly undetectable levels, whereas the Shp-1 band was only marginally decreased.

(Fig. 14, lane g). The levels of the endogenous cellular protein, Erp72, were equivalent in all of the transfected cells. These results indicate that the RID complex (i.e. RID α plus RID β), but not RID α or RID β alone, is sufficient to induce degradation of Fas.

A similar experiment was conducted except that cells were transfected with the
5 pcDNA3.1-CAT (InVitrogen, Carlsbad, CA) plasmid expressing chloramphenicol acetyl transferase (CAT) instead of pBUC-Shp-1. Since CAT is a bacterial protein, it is not possible for RID to have evolved in Ad to exert a specific biological effect on CAT. Expression of this protein was detected by immunoblot using anti-CAT antiserum obtained from 5 prime-3 prime. The results of the experiment were similar to those with Shp-1, i.e. Fas was greatly reduced in the presence of RID, whereas CAT was only marginally affected (Fig. 15).

10 These experiments demonstrate that the RID complex is sufficient to induce the internalization of cell-surface Fas into vesicles, presumably endosomes and lysosomes, to induce degradation of Fas, presumably in lysosomes, and to inhibit apoptosis triggered by an anti-Fas agonist monoclonal antibody.

15

Example 7

This example illustrates that RID inhibits killing of Ad-infected cells by natural killer cells and cytotoxic lymphocytes.

Natural killer (NK) cells and cytotoxic T-lymphocytes (CTL) play an important role
20 in the destruction of virus-infected cells during the early innate phase and the late immune-specific stages, respectively, of the host anti-viral response. Both NK and CTL kill targets via two major pathways. In one major pathway, perforin generates holes in the target and granzymes are introduced to induce apoptosis of the target cell. In another major pathway, Fas ligand on the surface of the CTL engages Fas on the target cell and induces apoptosis
25 through activation of the pro-apoptotic caspases. CTL can also kill cells through a third minor pathway, in which TNF expressed on the surface of CTL (or secreted by CTL) engages TNFR1 on targets and induces apoptosis via the caspases. In cell culture, TNF-mediated killing by CTL is observable in long term (> 24 h) killing assays. To investigate whether RID inhibits NK- and CTL-killing through Fas, the following experiments were conducted.

30 In the first experiment, which was described in the copending provisional application, the effect of Ad proteins on CTL-killing was assessed by performing a short-term CD3-dependent redirected cell assay (Azuma et al., *J. Exp. Med.* 175:353-360, 1992), using lymphocytes from perforin (-/-) mice (Kagi et al., *Science* 265:528-530, 1994) and from wild-type perforin (+/+) C57BL/6J mice acutely infected with influenza virus. Influenza virus
35 enhances the expression of Fas ligand in activated lymphocytes (Clark et al., *Immunol. Rev.* 146:33-44, 1995). In brief, mice were primed by intranasal infection of 50 HAU of HkX31

influenza A virus (Topham et al., *J. Virol.* 70:1288-1291, 1996; Tripp et al., *J. Immunol.* 154:6013-6021, 1995). CTL were isolated from the spleens of the infected mice, irradiated, and effector CTL generated by secondary *in vitro* re-stimulation. These CTL were further activated by incubation with the 145-2C11 anti-CD3ε mAb for 30 min on ice. Mouse Fas and 5 Fc receptor-positive P815 cells (1×10^6) were mock-infected or infected with 1000 PFU per cell of *rec*700 or *dl*7001 and labeled overnight with 100 μCi of $\text{Na}_2^{51}\text{CrO}_4$. These ^{51}Cr -labeled P815 target cells were washed, resuspended in DME, and then incubated with the activated anti-CD3ε-treated CTL using effector lymphocyte:target ratios of 60:1, 20:1 or 6:1. Cell lysis was determined 6 h later from a standard ^{51}Cr release assay and the results are 10 shown in Figs. 16A and 16B. The presence of Fas on the surface of P815 cells infected with *rec*700 or *dl*7000 was also examined by flow cytometry and the results are shown in Fig. 16C.

The perforin (-/-) CTL lysed mock-infected P815 cells efficiently (Fig. 16A). Lysis was inhibited by *rec*700 but not by *dl*7001 (lacks all E3 genes). Since the mice lack perforin, it follows that the CTL were killing the mock- and mutant-infected cells through the Fas 15 pathway and that the E3 region is required to inhibit killing through this pathway. The CTL from perforin (+/+) mice killed mock-, *rec*700-, or *dl*7001-infected P815 cells with similar high efficiency (Fig. 16B). Cell surface Fas was diminished on P815 cells infected with *rec*700 but not with *dl*7000 (lacks all E3 genes except for E3-14.7K) (Fig. 16C). These 20 results indicate that E3 proteins expressed by *rec*700 but not *dl*7000, presumably RID, inhibit CTL killing through the Fas pathway by down-regulating Fas from the cell surface.

A second experiment was conducted to investigate the role of RID in inhibiting killing of Ad-infected cells by NK cells. Human AS49 cells were mock-infected or infected with *rec*700 (wild-type Ad) or *dl*764, a virus mutant that lacks only RIDβ and then labeled with 100 μCi of $\text{Na}_2^{51}\text{CrO}_4$. These ^{51}Cr -labeled AS49 target cells were washed, resuspended 25 in DME, and then incubated with a semi-permanent line of human NK cells. After 24 h. cell lysis was measured based on release of ^{51}Cr from the cells as described elsewhere (Tollefson et al., *Nature* 392:726-730 (1998)) and the results are shown in Figure 17.

Mock-infected cells were lysed efficiently at NK:AS49 cell ratios of 10:1 and 5:1 (Fig. 17). This lysis was dramatically inhibited by infection with *rec*700, but it was only 30 marginally reduced by infection with *dl*764 (Fig. 17). Since the only protein not expressed by *dl*764 is RIDβ, it is believed that RID is required to inhibit killing of Ad-infected cells by NK cells. Most likely RID inhibits killing by NK cells by blocking the Fas pathway. However, a RID effect on the perforin-granzyme pathway cannot be excluded.

In summary, RID inhibits killing of Ad-infected cells by NK cells and by CTL. Thus, 35 RID should protect infected cells from attack by killer cells that are active in both the early

innate phase and the late immune-specific phase of the anti-viral immune response.

Similarly, transplanted cells and tissues are destroyed by NK cells and CTL. Therefore, RID should be useful to inhibit killing of transplanted cells or tissues by NK cells and CTL.

5

Example 8

This example illustrates that RID is required and probably sufficient to remove the TNFR1 from the cell surface.

Human HeLa cells were mock-infected or infected with 50 PFU/cell of *rec*700 (wild-type) or *d*712, which is a *rec*700-derived mutant with a deletion in the *adp* gene in the E3 region that results in overexpression of both RID (i.e. RID α and RID β) and E3-14.7K, and only trace amounts of other E3 proteins (Tollefson et al., *J. Virol.* 64:794-801, 1990; Tollefson et al., *Virol.* 175:19-29, 1990; Gooding et al., *Cell* 53:341-346, 1988). At 26 h p.i., cells were analyzed by flow cytometry (Tollefson et al., *Nature* 392:726-730 (1998)) using the B/O:2/18/91 rabbit antiserum against TNFR1 (obtained from Immunex Corp.) and PE-conjugated goat anti-rabbit IgG (Caltag). Fas was detected in the same experiment using supernatants from the M38 anti-Fas hybridoma cell line (obtained from the American Type Culture Collection) and FITC-conjugated goat anti-mouse IgG. The results are shown in Figure 18.

As shown in Fig. 18A, TNFR1 was removed from the surface of most cells infected with *rec*700 (red trace) or *d*712 (blue trace). The percentage of mock-infected cells that were stained for TNFR1 was 93%, as compared to 16% and 18%, respectively, for *rec*700 and *d*712. In this same experiment, cell surface Fas was also internalized by *rec*700 and *d*712 (Fig. 18B). Thus, Ad infection removes TNFR1 from the cell surface, as is the case with Fas.

The mutant used in the above experiment, *d*712, overexpresses RID and E3-14.7K, and expresses very little of the other E3 proteins. To determine whether RID and/or E3-14.7K is involved in internalization of TNFR1 in Ad-infected HeLa cells, the same experiment was performed using *d*712 and additional E3 mutants: *d*709, which lacks RID α , RID β , and E3-14.7K; *d*753, which lacks RID α but expresses RID β and E3-14.7K; and *d*764, which lacks RID β but expresses RID α and E3-14.7K. The deletions in these mutants do not affect expression of any other Ad proteins. The results are shown in Figure 19.

With *rec*700 and *d*712, TNFR1 was removed from the cell surface such that only 29% and 24%, respectively, of cells were stained for TNFR1 as compared to 92% with mock-infected cells (Fig. 19A). With *d*709, *d*753, and *d*764 infected cells, 84%, 85%, and 84%, respectively, were stained for TNFR1, indicating that these mutants did not induce removal of TNFR1 from the cell surface. Cell surface Fas was also examined in this same experiment.

rec700 and *dl712* cleared Fas whereas *dl309*, *dl753*, and *dl764* did not (Fig. 19B). Thus, RID is required to remove TNFR1 from the surface of Ad-infected cells, as is the case with Fas.

As a means to determine whether RID is sufficient to remove TNFR1 from the cell surface, HeLa cells were infected with the Ad vector named 231-10. This vector will be 5 described in detail in Example 10 below. In brief, 231-10 lacks the E1A, E1B, and E3 transcription units. The deleted E1A plus E1B regions are replaced with an expression cassette wherein all the E3 proteins are expressed from the human cytomegalovirus (CMV) promoter. Because 231-10 lacks E1A, viral genes in the vector backbone are not expressed; only the E3 proteins are expressed from the CMV promoter. Thus, the vector serves as an 10 essentially inert vehicle by which E3 genes can be delivered into cells and the properties of their proteins studied.

HeLa cells were mock-infected or infected with the 231-10 vector, and cell surface TNFR1 was examined by flow cytometry at 24 h and 48 h p.i. as described above. At 24 h p.i., the percentage of cells bearing TNFR1 was reduced from 93% to 35%, and by 48 h the 15 percentage was reduced to 11% (Fig. 20). This time course of TNFR1 down-regulation correlates with expression of the E3 proteins. In a parallel experiment, Fas was nearly completely cleared by 24, 36, and 48 h p.i. (data not shown). Thus, TNFR1 and Fas are removed from the cell surface by the E3 proteins expressed by 231-10. RID is undoubtedly the E3 protein responsible for the removal of these death receptors.

The ability of Ad and the RID protein to remove TNFR1 from the cell surface was 20 examined using the biotin-streptavidin system (Stewart et al., 1995) to detect TNFR1. Multiple dishes of A549 cells were mock-infected or infected with 50 PFU/cell of *rec700* (wild-type). At 16 h p.i., cell surface proteins in mock- and Ad-infected cells were labeled 25 using biotin. Ad-infected cells in other dishes were also labeled with biotin at 18, 20, 22, 24, and 30 h p.i. Proteins were extracted using buffer containing 0.5% NP-40, and were incubated with protein A-Sepharose CL-4B attached to the B/O:2/18/91 rabbit antiserum against TNFR1. After washing, proteins were solubilized, subjected to SDS-PAGE, and transferred to membranes. Membranes were incubated with peroxidase-conjugated streptavidin (Sigma), and proteins were visualized using ECL (Amersham).

In this assay, if Ad infection has resulted in the removal of TNFR1 from the cell 30 surface, then TNFR1 will not be available for biotinylation and therefore TNFR1 will not be detected. As shown in Fig. 21, similar amounts of TNFR1 were obtained from mock- or *rec700*-infected cells at 16 h p.i. With *rec700*, TNFR1 declined from 18 to 30 h p.i. until only small amounts were detected. Thus, as was the case when TNFR1 was detected by flow 35 cytometry, Ad infection results in markedly decreased amounts of cell surface TNFR1.

The ability of the 231-10 Ad vector to down-regulate cell surface TNFR1 as determined with the biotin-streptavidin assay was also examined. As discussed above, 231-10 expresses only Ad E3 proteins. Cells were mock-infected, infected with 50 PFU/cell of *rec700* (wild-type), or infected with 250 PFU/cell of 231-10. At different days p.i., cells were 5 biotinylated and TNFR1 detected as described above. As expected, most of the TNFR1 was cleared by *rec700* at 1 day p.i. (Fig. 22A, compare lanes a and b). With 231-10, reduced amounts of TNFR1 were detected by 1 day p.i., and by 5 days p.i. the TNFR1 levels declined to those of *rec700*. The levels of TNFR1 in mock-infected cells were similar after 5 days to those after 1 day (Fig. 22A, compare lane h with lane a). Therefore, the reduction at 5 days 10 seen with 231-10 is not due to a non-viral event associated with maintaining the cells in dishes for 5 days. These results indicate that the E3 proteins expressed by the 231-10 vector, presumably RID, are sufficient to clear TNFR1 from the cell surface.

The accumulation of RID β in these same cell extracts was also examined by standard immunoblot using the rabbit P118-132 antiserum (Stewart et al., 1995). With *rec700*, RID β 15 was abundant after 1 day (Fig. 22B, lane b). The multiple bands on RID β are species of RID β that are differentially O-glycosylated and phosphorylated. With 231-10, RID β was detected after 2 days, and it increased dramatically in abundance from days 3-5 (Fig. 22B, lanes c-g). Therefore, as expected, the accumulation of RID β in this experiment correlated inversely with the decline in cell-surface TNFR1.

20 These results obtained using the B/O:2/18/91 antibody in the biotin-streptavidin and flow cytometry assays to detect TNFR1 are consistent. Thus, it is believed that RID is necessary to efficiently down-regulate cell surface TNFR1 in Ad-infected cells. The results with 231-10 indicate that RID is sufficient to down-regulate TNFR1, with the caveat that the E3 14.7K and gp19K proteins, and possibly the E3 12.5K and 6.7K proteins, are expressed by 25 231-10.

To determine if RID is responsible for clearance of cell-surface TNFR1, the following Ad E3 mutants were used: *dl748*, which overexpresses RID β but lacks RID α ; and *dl798*, which overexpresses RID α but lacks RID β . A549 cells were mock-infected or 30 infected with 50 PFU/cell of *rec700*, *dl748*, or *dl798*, or infected with 25 PFU/cell each of *dl748* and *dl798*. At 26 h p.i. cells were biotinylated and TNFR1 examined as described above. As a positive control, a dish of mock-infected cells was treated with TNF, and the cell extract was examined for TNFR1. As expected, TNF removed most of the TNFR1 from the cell surface (Fig. 23A, lanes a and b).

35 The results with the viruses are shown in Fig. 23A, lanes c-f. With *rec700* (wild-type)-infected cells, only small amounts of TNFR1 were detected (lane c). With *dl748*

(RID α -, RID β +) and d798 (RID α +, RID β -), high to intermediate levels of TNFR1 were observed, indicating that RID α and RID β are required for efficient clearance of TNFR1. When cells were co-infected with d748 and d798, TNFR1 was reduced to levels comparable to rec700-infected cells (lanes f and c). This result indicates that the mutants complement (d748 provides RID β , d798 provides RID α), and that both RID α and RID β are required for efficient removal of TNFR1 from the cell surface. Figure 23B shows a standard immunoblot for E1B-19K from the same extracts that were analyzed for biotinylated TNFR1. Similar amounts of E1B-19K were detected with all viruses. Therefore, differences in TNFR1 levels seen with these viruses are not due to differences in infection efficiency by the viruses.

The partial clearance of TNFR1 observed with these RID α - and RID β - mutants is consistent with the flow cytometry data in Fig. 19. These results suggest that there may be a mechanism in addition to RID that down-regulates cell-surface TNFR1 in Ad-infected cells. However, clearly, most of the down-regulation of TNFR1 requires RID.

In summary, RID is required to remove TNFR1 from the surface of Ad-infected cells. RID is also sufficient for removal of TNFR1 as indicated by the experiment with the 231-10 vector, with the caveat that the 231-10 vector also expresses other E3 proteins. RID expressed by the 231-10 vector is also sufficient to remove Fas from the cell surface, again, with the same caveat. However, the down-regulation of TNFR1 and Fas by 231-10 is almost certainly due to RID, because the mutant mapping data with E3 mutants have provided no indication that other E3 proteins play any role in down-regulating these death receptors.

Example 9

This example demonstrates that the 231-10 vector prevents rejection of human cancer cells transplanted into immunocompetent mice.

25 Cells or tissues transplanted into immunocompetent recipients are usually destroyed
(rejected) by immune killer cells of the recipient. Rejection begins within 1-2 days, and
therefore is mediated by the innate immune system including macrophages and NK cells.
Specific CTL formed after about 5-7 days also play a major role in transplant rejection. As
discussed above in Example 7, RID inhibits NK- and CTL-killing of Ad-infected cells and
thus should also be able to inhibit NK- and CTL-mediated rejection of transplanted cells or
tissues.

This idea was tested by determining whether the E3 proteins expressed by the 231-10 vector will permit human cancer A549 cells to grow as a tumor in immunocompetent C57BL/6 (H-2^b) mice. Human cancer cells normally will be rejected when transplanted in

C57BL/6 mice. However, RID should inhibit rejection by removing Fas and TNFR1 from the transplanted cells. E3-14.7K may also prevent rejection.

A549 cells mock-infected or infected with 50 PFU/cell of 231-10. After 48 h, 2×10^6 cells (in 100 μ l) were injected subcutaneously into each hind limb flank of female C57BL/6 mice. At 18 days post-injection, the mice were sacrificed and the site of injection was examined following removal of the skin. With mice that received mock-infected cells, there was a pin-point mass on one flank, and no mass at all on the other flank (data not shown). With the 231-10-infected cells, there were significant tumor masses on both flanks (Fig. 24). The tumors were opaque and ellipsoid in shape. The left-flank tumor was attached to the muscle. The right-flank tumor, which is shown in higher magnification in Fig. 25, was attached to both the muscle and skin. The size of the tumor obtained with 231-10-infected cells was many times larger than what would be observed from the initial bolus of cells injected (2×10^6 cells are barely visible to the naked eye). Thus, the cells grew into a tumor.

In the second experiment, mock-infected and 231-10-infected A549 cells (at 2 days p.i. in culture, 50 PFU/cell) were used, both live cells as well as cells that were killed by freezing and thawing. These cells were injected into each hind limb of C57BL/6 and Balb/c mice, 2×10^7 cells per injection. As is the case with C57BL/6, the Balb/c mice are fully immunocompetent. There were four mice of each strain. Mouse 1 received killed uninfected A549 cells, mouse 2 received live A549 cells, mouse 3 received killed 231-10-infected cells, and mouse 4 received live 231-10-infected cells. Mice were harvested at 15 days following injection. No tumors were observed in either mouse strain with killed cells. With the C57BL/6 mouse that received uninfected live cells, there was no growth on one flank and a very small mass on the other flank. With the Balb/c mouse that received live uninfected cells, there were small flat masses on each flank. However, with both the C57BL/6 and the Balb/c mouse that received 231-10-infected cells, there were much larger ellipsoid masses (tumors) on both hind flanks. These tumors resembled the tumors shown in Figs. 24 and 25. Therefore, as was the case in the first experiment, the 231-10 vector allowed A549 cells to form tumors in immunocompetent mice.

One of the 231-infected cell tumors from the C57BL/6 mouse was examined for expression of the E3 proteins known to be synthesized in cultured cells by 231-10. Proteins were extracted from the tumor, and the RID β , 14.7K, and gp19K proteins assayed by immunoblot. As shown in Fig. 26, all three proteins were detected. This result provides very strong evidence that the cells originally infected with 231-10, at the very minimum, persisted in the mouse. It is very likely that these cells grew as well, considering that tumors were formed. It is not likely that the 231-10 vector replicated in these cells, because the vector

lacks the E1A gene. Most likely, as the A549 cells proliferated in the mouse, a portion of the input vector was segregated into the daughter cells.

In summary, the E3 proteins expressed from the 231-10 vector have permitted the growth of human A549 cancer cells to form tumors in C57BL/6 and Balb/c mice. The tumors would not have been able to form unless they were protected from destruction by the immune system. These results argue strongly that the E3 proteins should prevent immune rejection of other types of transplanted cells and tissues. Thus, the 231-10 vector has the potential to be used in tissue or cell transplants to prevent rejection of the tissues or cells.

Example 10

This example illustrates the construction and properties of the 231-10 vector.

Features of 231-10

The 231-10 vector is a human adenovirus serotype 5 (Ad5) vector. It can be viewed as a "transient transfection" system, analogous to that obtained when a plasmid expression vector is transfected into cells. The basic features of the 231-10 vector are outlined in the schematic shown in Fig. 27 and the entire DNA sequence of the genome of 231-10 is given in Fig. 28.

The horizontal bar in Fig. 27 depicts the linear double-stranded DNA genome. The base pairs (nucleotides) are numbered from 1 to 34427 (see Fig. 28), from left to right in Fig. 27. Nucleotides 342-3523 are deleted, removing all the genes in the Ad E1A and E1B transcription units (collectively known as E1). Nucleotides 28133-30818 are also deleted, removing all the genes in the E3 transcription unit. In place of E1, an expression cassette has been inserted, in which the E3 genes are expressed from the human cytomegalovirus immediate early promoter-enhancer (CMV). This E3 expression cassette contains the E3 genes from the virus named *pm734.1*, which is a derivative of the virus named *rec700* (Tollefson et al., *Virology* 220:152-162, 1996). *rec700* is an Ad5-Ad2-Ad recombinant that has the Ad2 version of the E3 genes for the 12.5K, 6.7K, gp19K, and RID α proteins, and the Ad5 version of the E3 genes for the RID β and 14.7K proteins. The E3 cassette in 231-10 contains all the E3 genes from *pm734.1*. Notably, there are two missense mutations in the *adp* gene (which encodes the Adenovirus Death Protein [ADP], previously named E3-11.6K) (Tollefson et al., *supra*). These two mutations eliminate the first two methionine codons in the *adp* gene, thereby precluding synthesis of functional ADP (Tollefson et al., *supra*).

The 231-10 vector was designed to have the following properties. First, since the E1A genes are lacking, the vector should not replicate (efficiently) on most cell lines. Therefore, Ad early and late proteins will not be expressed and Ad DNA will not replicate. (It is known that Ad mutants lacking E1A do replicate their DNA and express late proteins at low levels when high multiplicities of infection are used and the infection is allowed to

proceed for several days. This is also true for 231-10 [not shown].) Second, the E3 proteins should be expressed in an E1A-independent manner from the CMV promoter/enhancer. Thus, 231-10 is an essentially inert vehicle that can deliver the Ad E3 proteins into cells without having other Ad proteins expressed, at least for the first approximately 3 days 5 following infection. Even after 3 days, other Ad proteins should be expressed only in very small amounts, much less than the E3 proteins.

Construction of Ad 231-10

(a) The genes of the E3 transcription unit were excised from *pm734.1* (*pm734.1* is *rec700* with mutations of the Met1 and Met41 codons in the *adp* gene. *rec700* is the same as 10 *Ad5* but with the *Ad2 EcoRI-D* fragment substituted for the corresponding *Ad5 EcoRI-C* fragment). The *pm734.1 SrfI-NdeI-D* fragment (3560 bp) was blunt-end using the Klenow enzyme and cloned into the *SmaI* site of the *pBluescriptSK(+)* vector (Stratagene), resulting in plasmid *p1721* which has the whole E3 transcription unit of *pm734.1* (-39 to 3521) flanked by *Sall-BstXI-SacII-NotI-XbaI-SpeI-BamHI* sites situated upstream from the E3 sequences 15 and *PstI-EcoRI-EcoRV-HindIII-ClaI-SalI-XhoI* sites situated downstream from the E3 sequences.

(b) The *BamHI-SalI-A* fragment (3605 bp) of *p1721* was subcloned between the *BamHI-XhoI* sites of plasmid *pCDNA3.1zeo(+)* (Invitrogen), resulting in plasmid *p181* in which E3 genes are under control of the CMV promoter-enhancer.

(c) The *MfeI-ClaI* fragment of *p181* (4328 bp), corresponding to the CMV promoter-E3 genes from the *pm734.1* expression cassette, was subcloned between the *EcoRI-ClaI* sites of plasmid *pΔE1splA* (Microbix Biosystems Inc., Toronto), resulting in plasmid *p231* which has the CMV-E3 expression cassette flanked by *Ad5* genomic sequences (*Ad5* map units 0-1 and 9.8-16.1). The orientation of the CMV-E3 expression cassette is right-to-left (opposite to 25 the *Ad E1* and major late transcription units).

(d) Plasmid *p231* was cotransfected along with plasmid *pBHG10* (Microbix Biosystems Inc., Toronto) into 293 cells resulting in plaques of recombinant virus 231-10. The virus has deletions of *E1* (*Ad5* nt 342-3523) and *E3* (*Ad5* nt 28133-30818), and has the CMV-E3 expression cassette in place of the *E1* deletion.

30 The 231-10 Vector Expresses the E3 RID, 14.7K, and gp19K proteins.

The E3 proteins are expected to be synthesized from the E3 expression cassette in 231-10. To demonstrate that this is so, separate dishes of A549 cells were infected with 250 PFU/cell of 231-10, then at 0-5 days p.i. protein extracts were examined for the E3 RID, 14.7K, and gp19K proteins using standard immunoblot procedures (Tollefson et al., Nature 35 392:726-730 (1998)). In one dish, 231-10-infected cells were treated with 1-β-D-

arabinofuransylcytosine (araC) at 2 h p.i., then proteins were extracted at 1 day p.i. RID β , 14.7K, and gp19K were readily detected at 2 days p.i., and their abundance increased until the end of the experiment at 5 days p.i. (Fig. 29, lanes d-g). On longer exposures of the gel shown in Fig. 29, a trace of RID β , 14.7K, and gp19K can be seen at 1 day p.i. (not shown).

5 In the experiment shown in Fig. 29, one dish of cells was treated with araC. AraC inhibits Ad DNA replication, and therefore Ad late genes cannot be expressed. As shown in Fig. 29, small amounts of RID β and gp19K were detected in the araC-treated cells; 14.7K was also detected in longer exposures of the gel (lane A). Therefore, as expected, E3 proteins are synthesized by 231-10 without replication of the vector Ad DNA.

10 These results demonstrate that the RID β , 14.7K, and gp19K proteins are expressed in 231-10-infected cells. In another experiment, the levels of RID β at 4 or 5 days p.i. were roughly similar to those of rec700-infected cells at 1 day p.i. (see Fig. 22). Bearing in mind that rec700 has replicated by 1 day p.i. and therefore has expressed higher levels of RID β from more templates, the quantities of RID β , 14.7K, and gp19K observed with 231-10, which
15 does not replicate (or only replicates in small amounts at 4 or 5 days p.i.), are quite high. The synthesis of the E3 12.5K and 6.7K proteins by 231-10 has not been examined. Although not shown directly in Fig. 29, the RID α polypeptide is also expressed by 231-10. This can be deduced from the observation that 231-10 exhibits the expected functions of RID, namely it
20 clears Fas and TNFR1 from the surface of infected cells (see Example 8.). These functions require both RID α and RID β .

Indirect immunofluorescence was also used to study the expression of the gp19K, RID β , and 14.7K proteins in A549 cells infected with 231-10. At 2 days p.i., the gp19K and RID β proteins were visualized as described previously (Tollefson et al., *Nature* 392:726-730 (1998); Hermiston et al., *J. Virol.* 67:5289-5298 (1993)) and the 14.7K protein was stained
25 using a rabbit antiserum directed against a TrpE-14.7K fusion protein (Tollefson and Wold, *J. Virol.* 62:33-39 (1988)). Strong staining of gp19K was observed in a pattern typical of the endoplasmic reticulum (Fig. 30A), as has been observed with rec700 (Hermiston et al., *supra*). The pattern for RID β was also similar to that seen with rec700, i.e. staining of the Golgi, other membranes, and the plasma membrane (Fig. 30B; Tollefson et al., *Nature*
30 392:726-730 (1998)). The 14.7K protein staining was diffuse in the cytoplasm (Fig. 30C), which again is typical of rec700 (unpublished results). These results establish that the E3 gp19K, RID, and 14.7K proteins localize to the same or similar intracellular compartments as they do in wild-type Ad-infected cells.

In view of the above, it will be seen that the several advantages of the invention are
35 achieved and other advantageous results attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

5 All references cited in this specification are hereby incorporated by reference. The discussion of references herein is intended merely to summarize the assertions made by their authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinency of the cited references.

What is Claimed is:

1. A method for inhibiting apoptosis of a cell comprising treating the cell with an effective amount of a Receptor Internalization and Degradation (RID) complex.
2. The method of claim 1 wherein the treating step comprises administering to the cell a polynucleotide encoding the RID complex and wherein the RID complex is expressed in the cell.
3. The method of claim 2 wherein the polynucleotide comprises a recombinant adenovirus vector.
4. The method of claim 3 wherein the recombinant adenovirus vector is 231-10.
5. The method of claim 3 wherein the cell expresses Fas, TNFR-1, DR3, TRAIL-R1, or TRAIL-R2.
6. The method of claim 5 wherein the cell is a leukocyte.
7. The method of claim 5 wherein the cell comprises a transplant tissue.
8. The method of claim 1 wherein the treating step comprises administering the RID complex to the cell.
9. The method of claim 8 wherein the RID complex is administered with a carrier which facilitates delivery of the RID complex into the cell.
10. A method for decreasing apoptosis of target cells in a patient comprising treating the patient with an effective amount of a Receptor Internalization and Degradation (RID) complex.
11. The method of claim 10 wherein the treating step comprises administering to the patient a polynucleotide encoding the RID complex and wherein the polynucleotide is internalized in the target cells and the RID complex is expressed.
12. The method of claim 11 wherein the polynucleotide comprises a recombinant adenovirus vector.
13. The method of claim 12 wherein the recombinant adenovirus vector is 231-10.
14. The method of claim 10 wherein the patient suffers from a degenerative disease or an immunodeficiency disease.
15. The method of claim 10 wherein the treating step comprises administering the RID complex to the patient.
16. The method of claim 15 wherein the RID complex is administered with a carrier which facilitates delivery of the RID complex into the cells.
17. A method for decreasing leukocyte apoptosis in a patient comprising:
 - (1) withdrawing leukocytes from the patient,
 - (2) treating the leukocytes with an effective amount of a RID complex, and
 - (3) administering the treated leukocytes to the patient.

18. The method of claim 17 wherein the treating step comprises administering to the leukocytes a polynucleotide encoding the RID complex wherein the RID complex is expressed in the leukocytes.
19. The method of claim 18 wherein the polynucleotide comprises a recombinant adenovirus vector.
20. The method of claim 19 wherein the recombinant adenovirus vector is 231-10.
21. The method of claim 17 wherein the treating step comprises administering the RID complex to the leukocytes.
22. The method of claim 21 wherein the RID complex is administered with a carrier which facilitates delivery of the RID complex into the leukocytes.
23. A composition comprising a Receptor Internalization and Degradation (RID) complex and a carrier suitable for facilitating delivery of the RID complex into a cell.
24. A recombinant adenovirus comprising a polynucleotide encoding a Receptor Internalization and Degradation (RID) complex operably linked to a promoter, wherein the adenovirus is replication defective and wherein the polynucleotide is expressed upon infection of a eukaryotic cell with the adenovirus.
25. The recombinant adenovirus vector of claim 24 consisting of 231-10.

1/85

TNFR1	362	V V E N W P L R W K I R G C E S D H E	R I T E L O N G R C L P E A Q W	401
Fas	236	I A G V M T L S Q V N G Y E E E	E E V E V E I G R - F F E Q Q	275
DR3	338	V M D A V E P A R R V K E T M C U Y	R E A E E F A V E R A G T A G - P G D A L	376
TRAIL-R1	348	F A N I V P P D S W D Q L M Q D	M D N E T K N E T K N E A A G - H E D T L	386
TRAIL-R2	316	F A D L V P P D S W E P L M Q D	M D N E T K N E T K N E A A G - H E D T L	354
TNFR1	402	S M L A T M R R R P P R E A T W E L E	G R V E R D M D L L G C L D E E	439
Fas	276	Q L L R N H Q R - K E A Y D T L I K D	K K A N C T L A E K Q T	313
DR3	377	E M E K R V R - Q Q Q - P A G M G A V Y A V E E M G	D G C V E D I L R S	411
TRAIL-R1	387	A M E M V V K V G R - N A S E V Y L E D A T E R M E E R	H A K E K I Q D	423
TRAIL-R2	356	T M I V V K V G R - D A S E V Y L E D A T E R M E E R	L A K Q K I E D	392

Figure 1

2 / 85

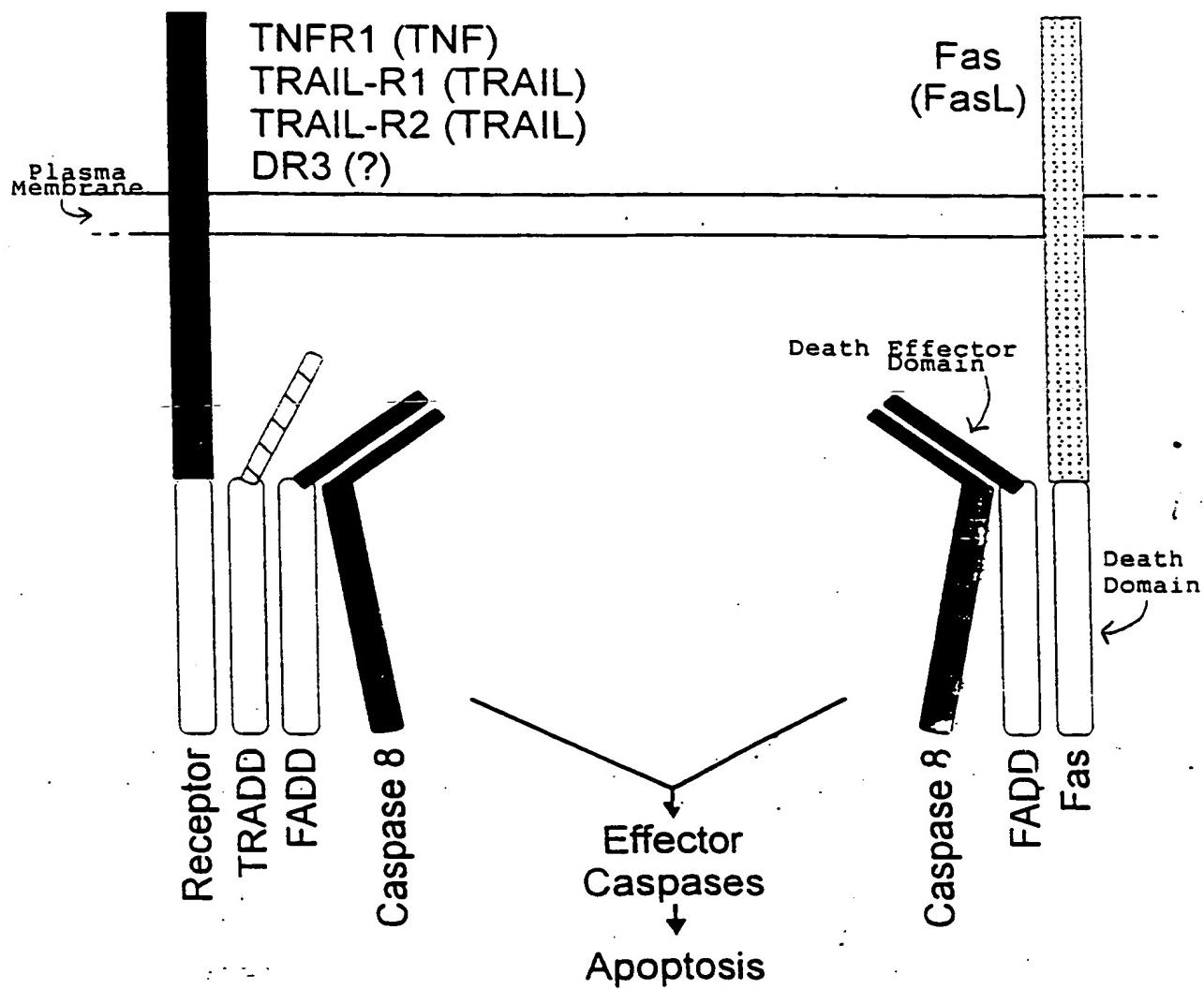


FIGURE 2

3/85

RID COMPLEX

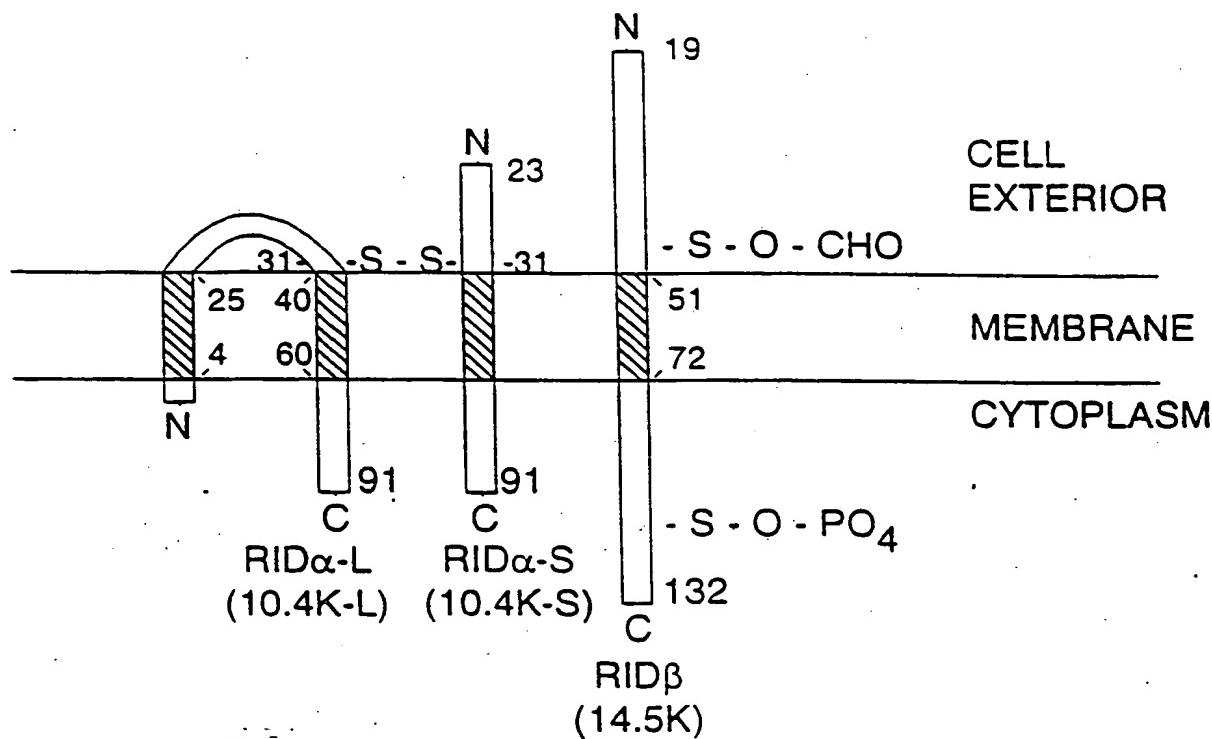


Figure 3

4 / 85

RID α -L (10.4K-L)

M I P R V L I L L T L V A L F C A C S T L A A V A H I E
 signal sequence

10 20
 V D C I P P F T V Y L L Y G F V T L I L I C S L V T V V
 * transmembrane

30 40 50
 I A F E I Q F I D W V C V R I A Y L R H H P Q Y R D R T I

60 70 80
 A D L L R I L

Figure 4A

RID α -S (10.4K-S)

A V A H I E V D C I P P F T V Y L L Y G F V T L I L I C
 * transmembrane
 S L V T V V I A F I Q F I D W V C V R I A Y L R H H P Q
 Y R D R T I A D L L R I I

Figure 4B

5 / 85

Pre-RID β (14.5K)

10 20
M K F T V T F L L I I C T L S A F C S P T S K P Q R H I
signal sequence

30 40 50
S C R F T R I W N I P S C Y N E K S D L S E A W L Y A I

60 70 80
I S V M V F C S T I L A L A I Y P Y L D I G W N A I D A
Transmembrane

90 100 110
M N H P T F P A P A M L P L Q Q V V A G G F V P A N O P

120 130
R P P S P T P T E I S Y F N L T G G D D

Figure 4C

Mature-RID β (14.5K)

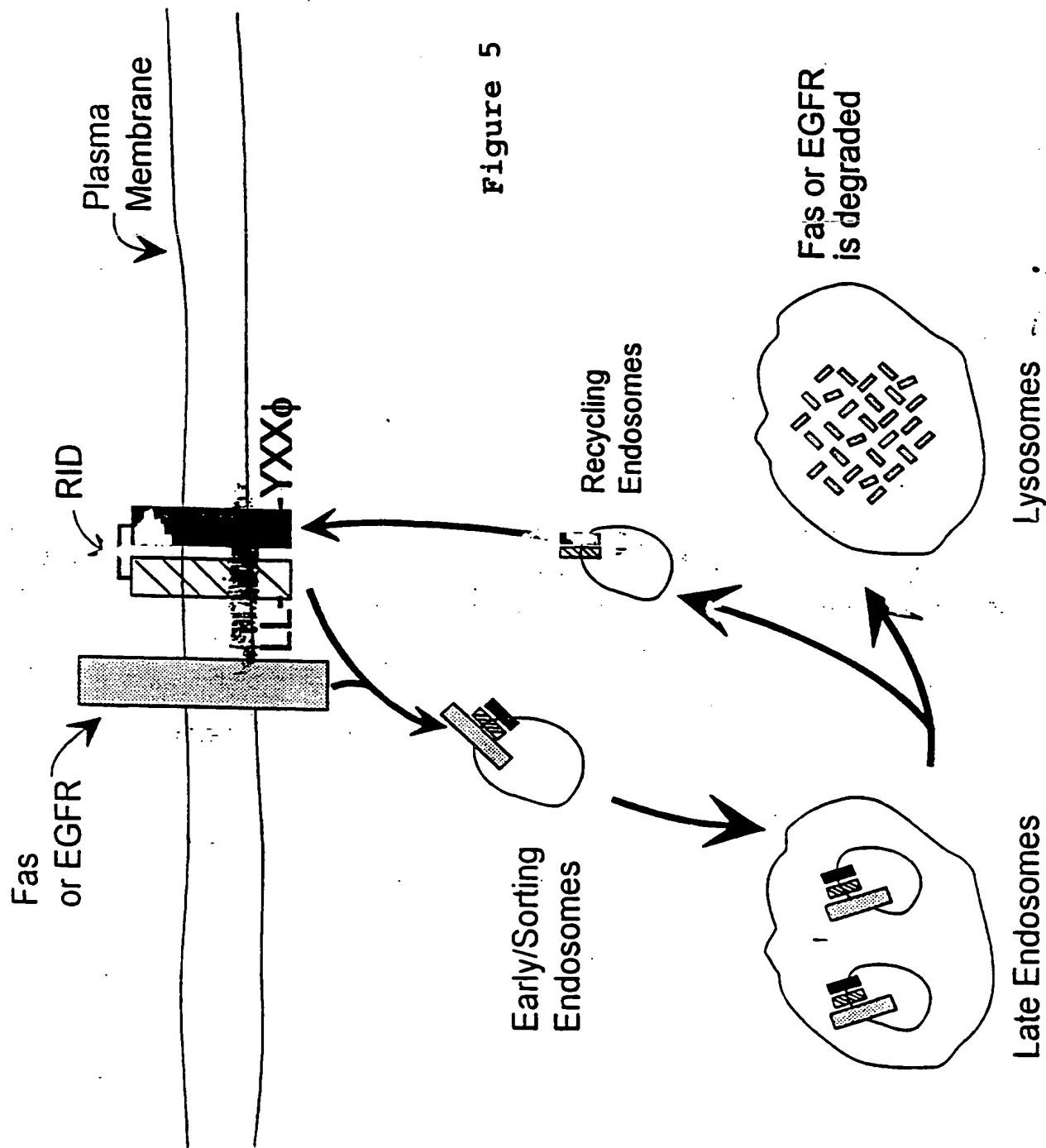
10 20
S P T S K P Q R H I S C R F T R I W N I P S C Y N E K S

30 40 50
D L S E A W L Y A I I S V M V F C S T I L A L A I Y P Y
Transmembrane

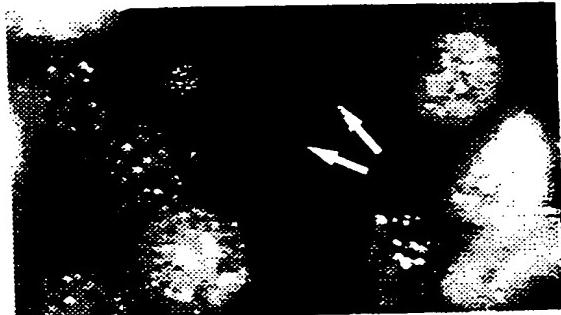
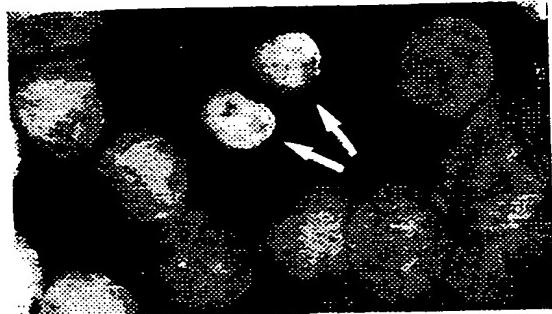
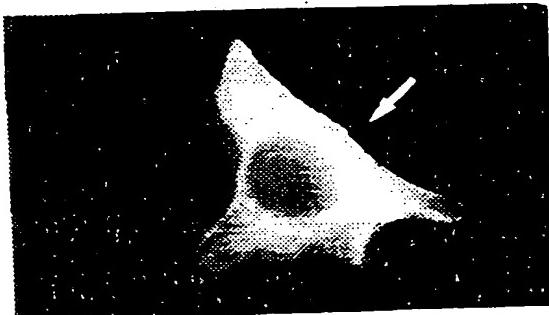
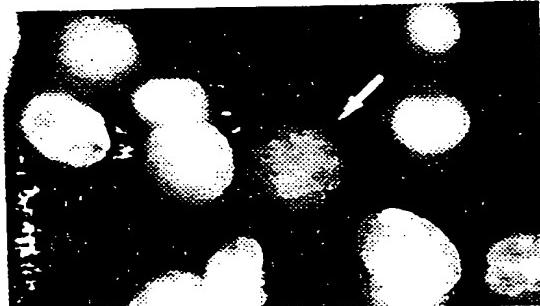
60 70 80
L D I G W N A I D A M N H P T F P A P A M L P L Q Q V V

Figure 4D

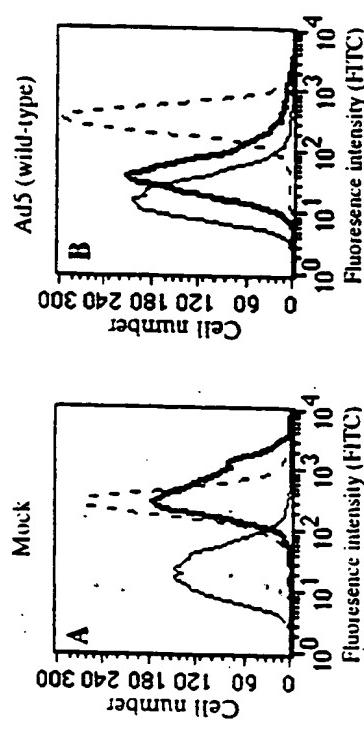
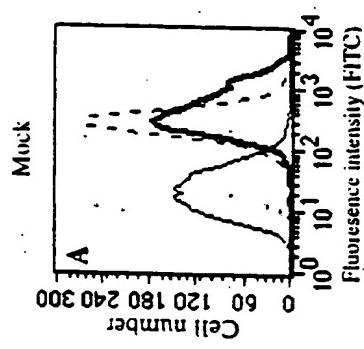
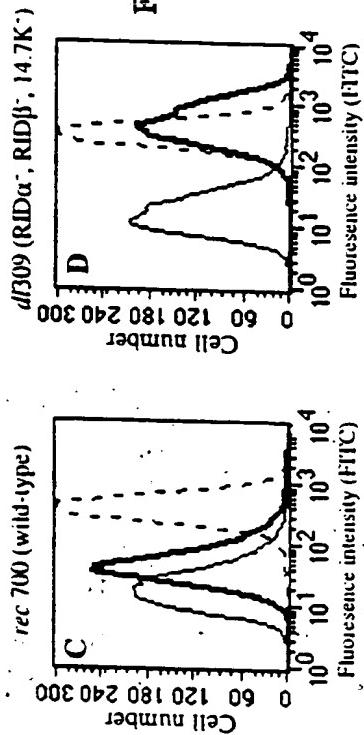
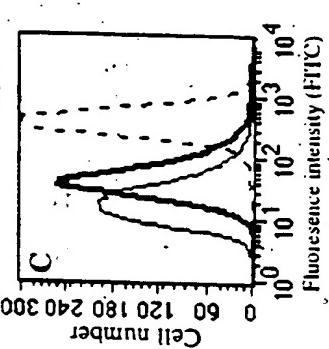
6 / 85



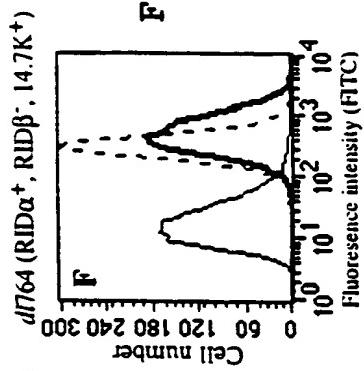
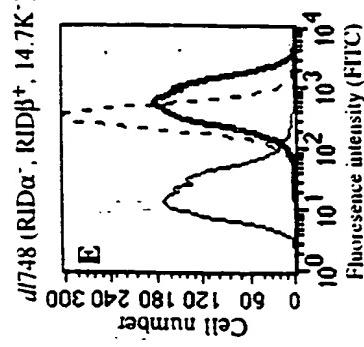
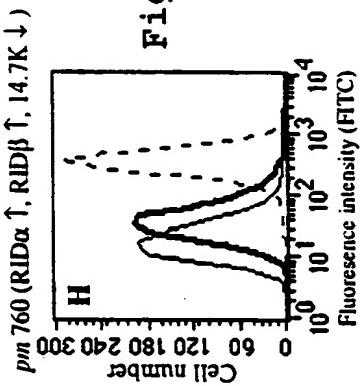
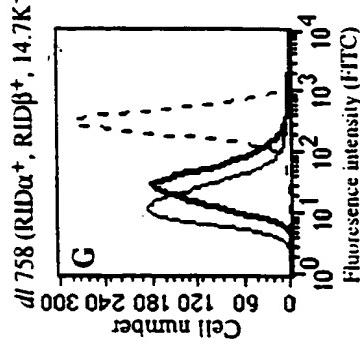
7/85

rec700, anti-DPB**Figure 6A***rec700, DAPI***Figure 6B***RID, anti-RID β* **Figure 6C***RID, DAPI***Figure 6D**

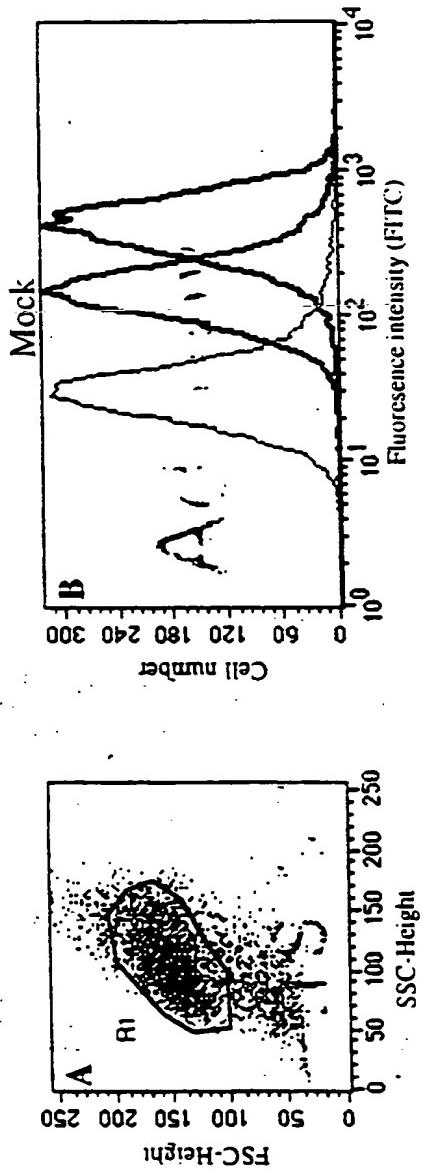
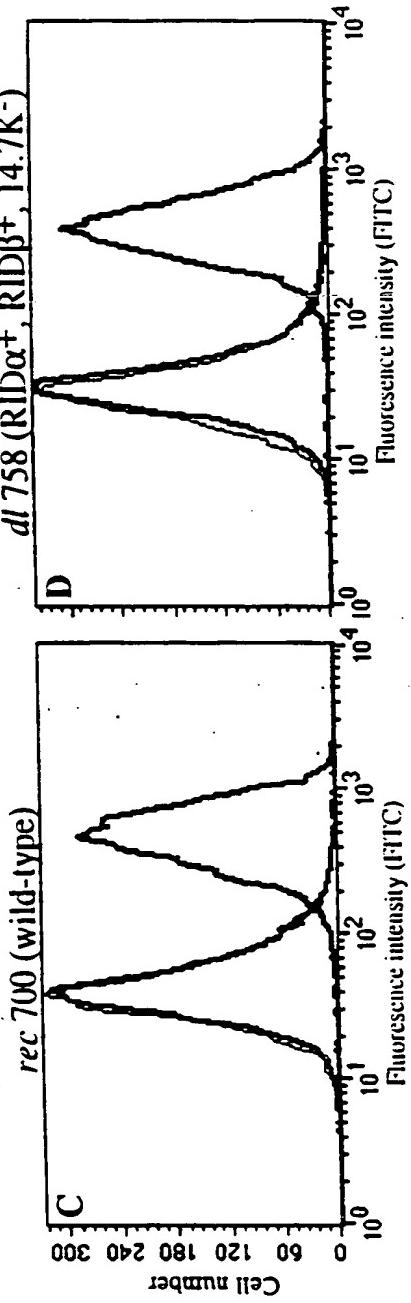
8 / 85

Figure 7B**Figure 7A****Figure 7D****Figure 7C**

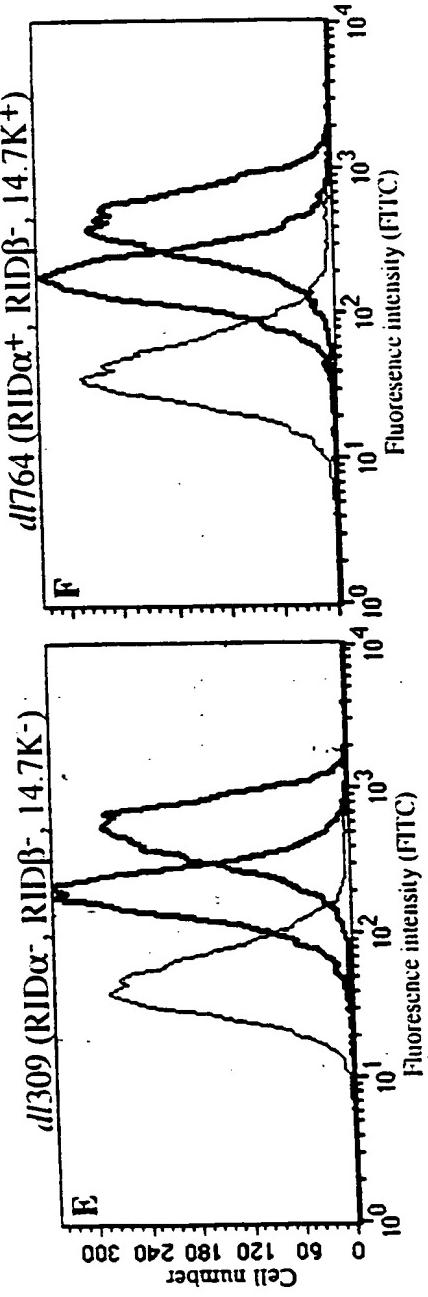
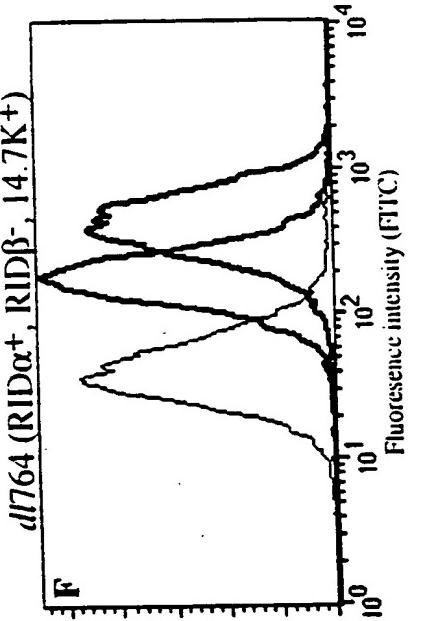
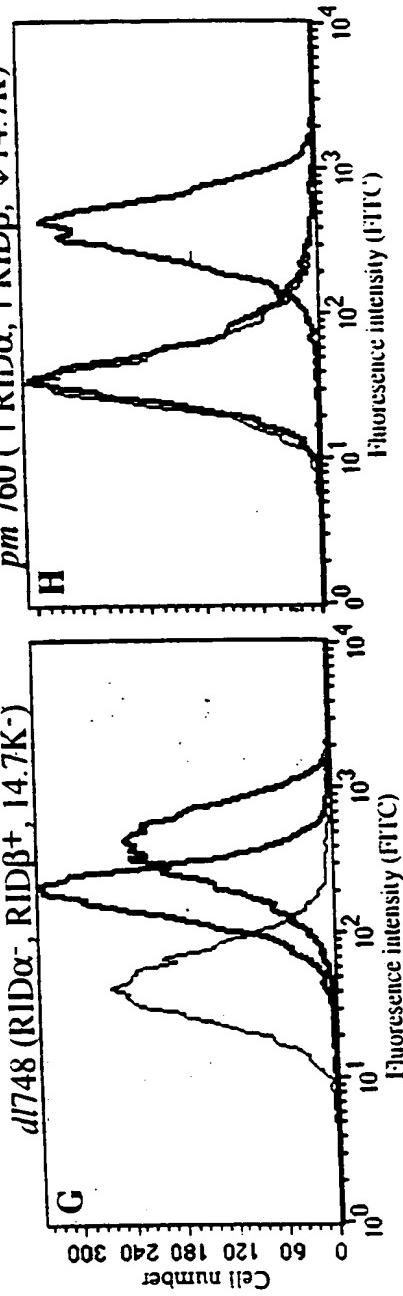
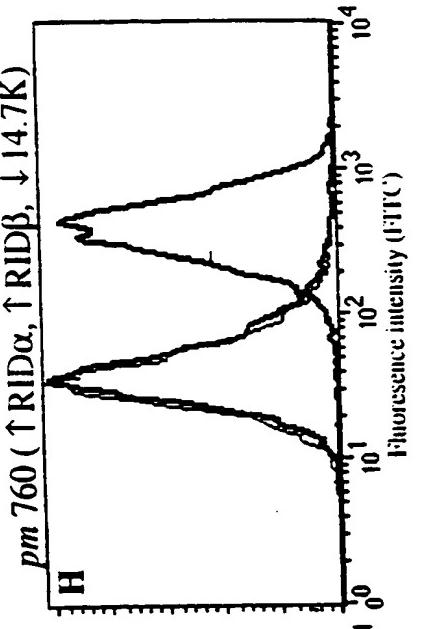
9 / 85

Figure 7F**Figure 7E****Figure 7H****Figure 7G**

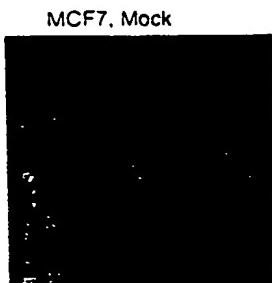
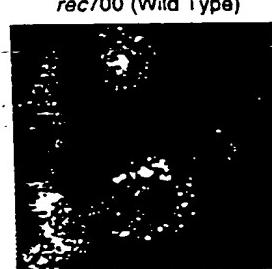
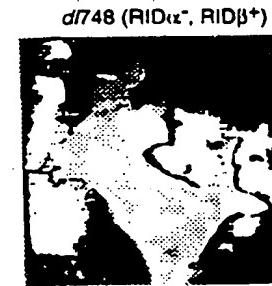
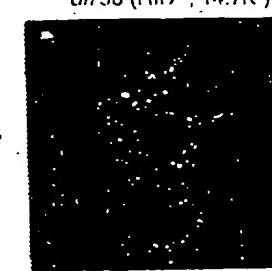
10 / 85

Figure 8B**Figure 8C****Figure 8D**

11 / 85

Figure 8E**Figure 8F****Figure 8G****Figure 8H**

12 / 85

Figure 9A**Figure 9B****Figure 9C****Figure 9D****Figure 9E****Figure 9F****Figure 9G****Figure 9H**

13 / 85

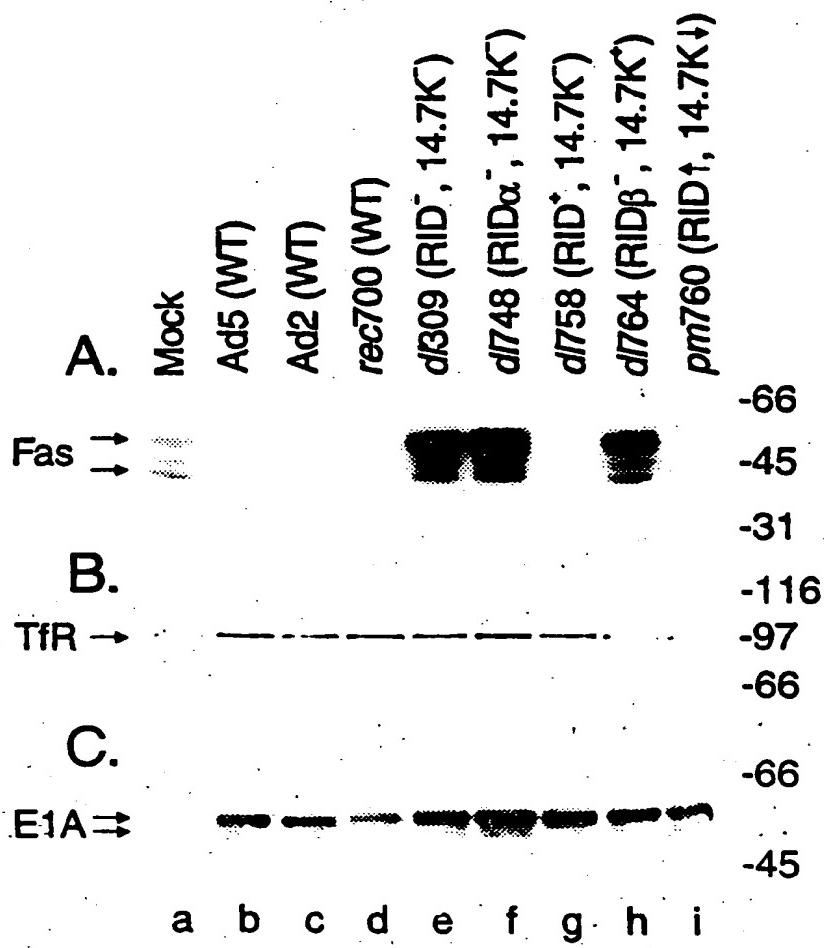


Figure 10

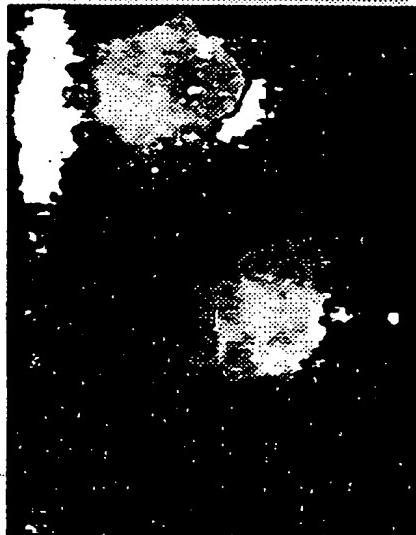
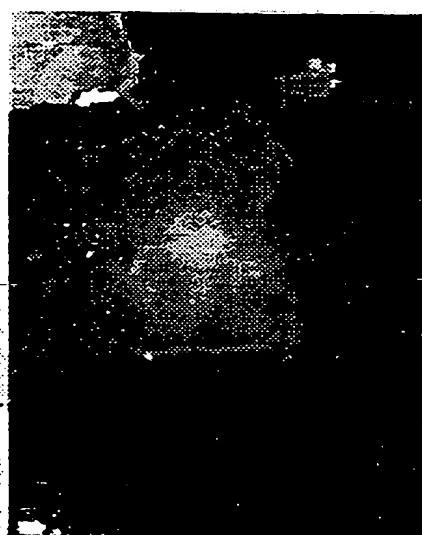
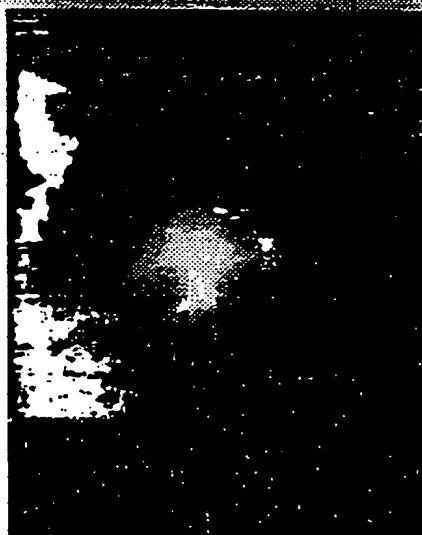
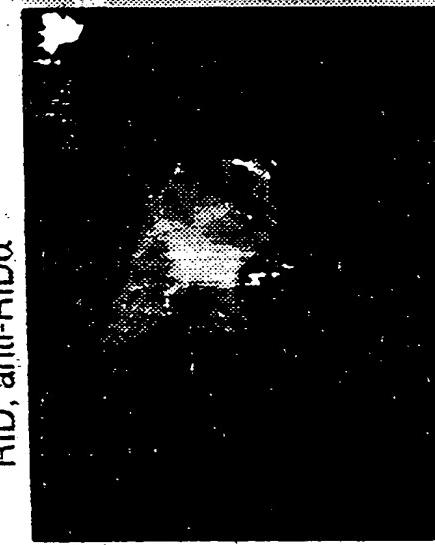
Figure 11BR $\text{ID}\alpha$, anti-Fas**Figure 11A**R $\text{ID}\alpha$, anti-R $\text{ID}\alpha$ **Figure 11D**R $\text{ID}\beta$, anti-Fas**Figure 11C**R $\text{ID}\beta$, anti-R $\text{ID}\beta$ 

Figure 11F

RID, anti-RID α

Figure 11E



RID, anti-Fas

Figure 11H

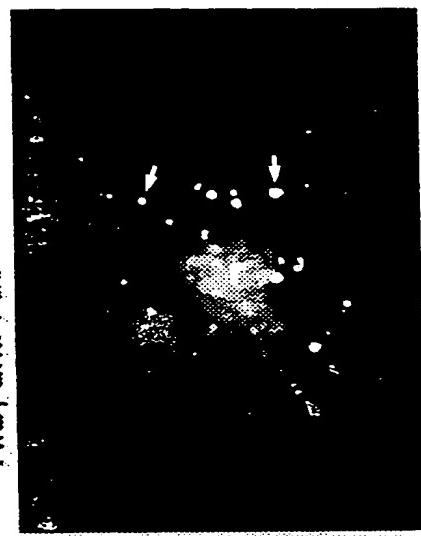
RID, anti-RID β

Figure 11G



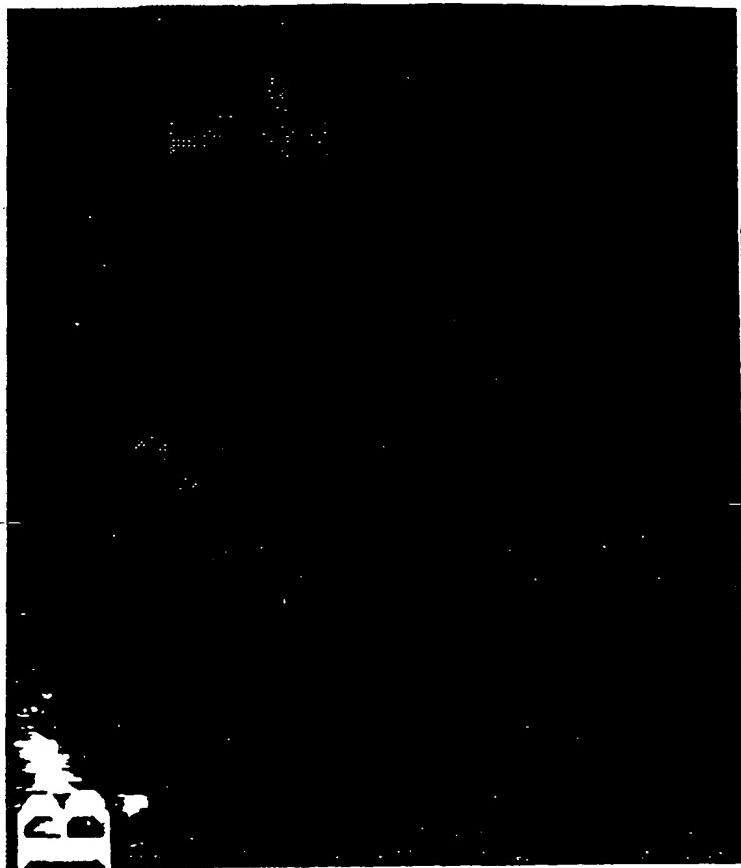


Figure 12B

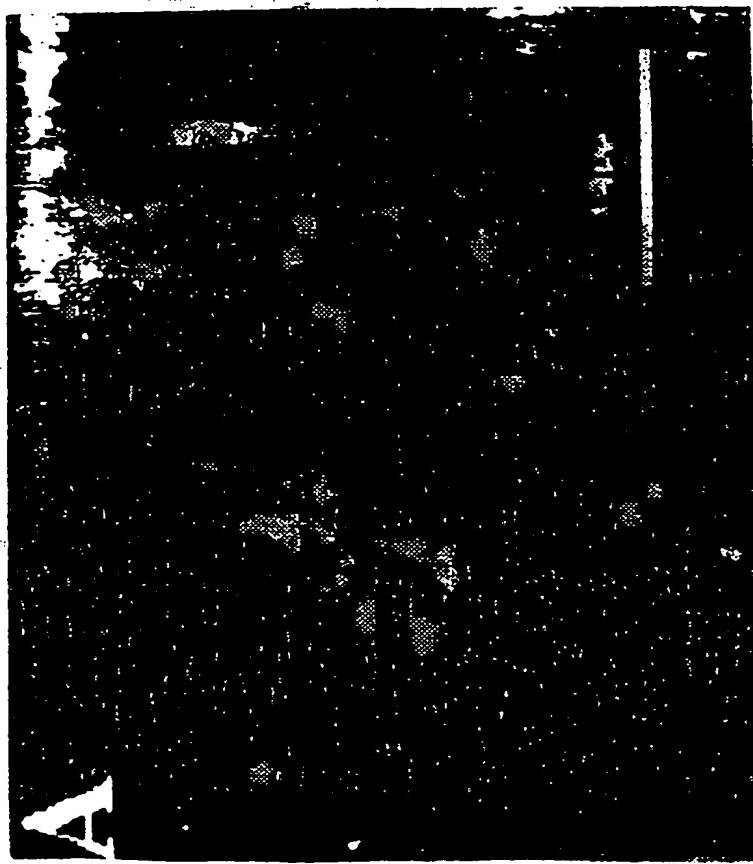


Figure 12A

17/85

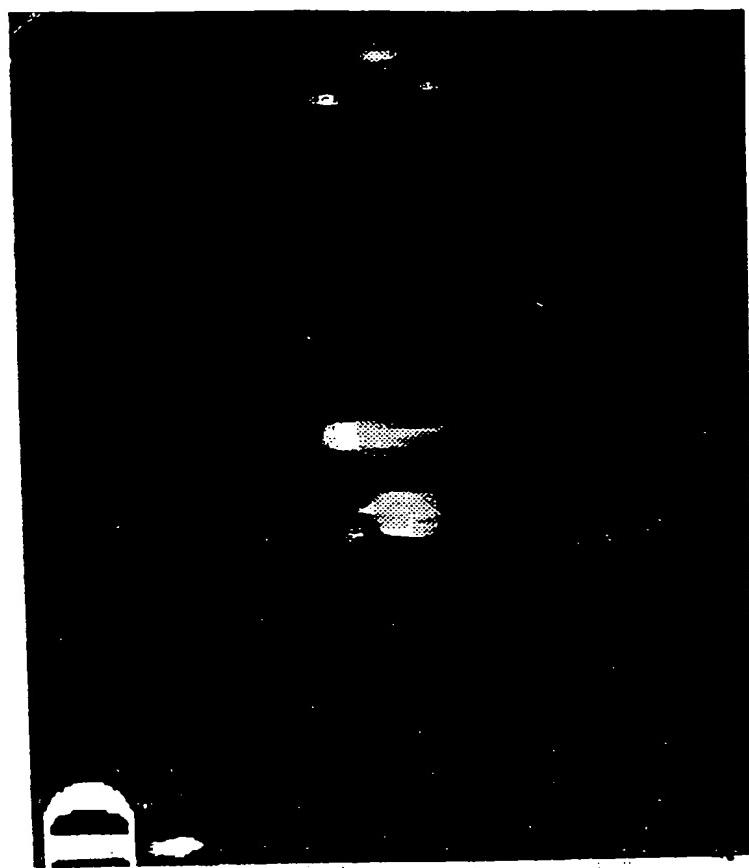


Figure 12D

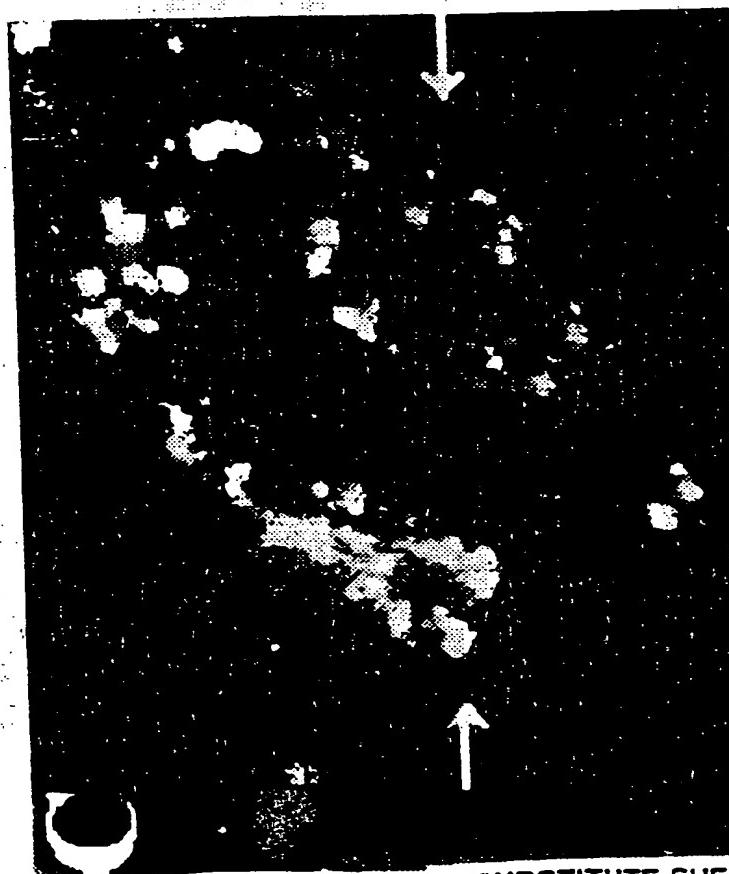


Figure 12C

18/85

rec700 (WT), Baf⁺

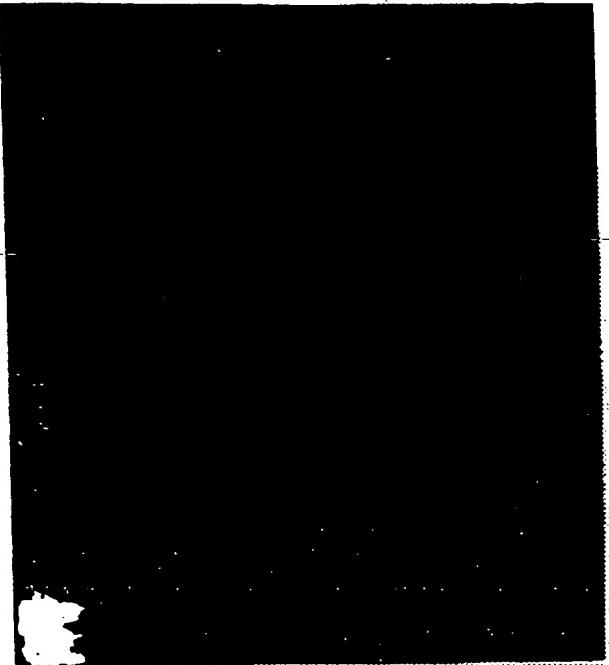


Figure 13B

rec700 (WT), Baf⁻



Figure 13A

19/85

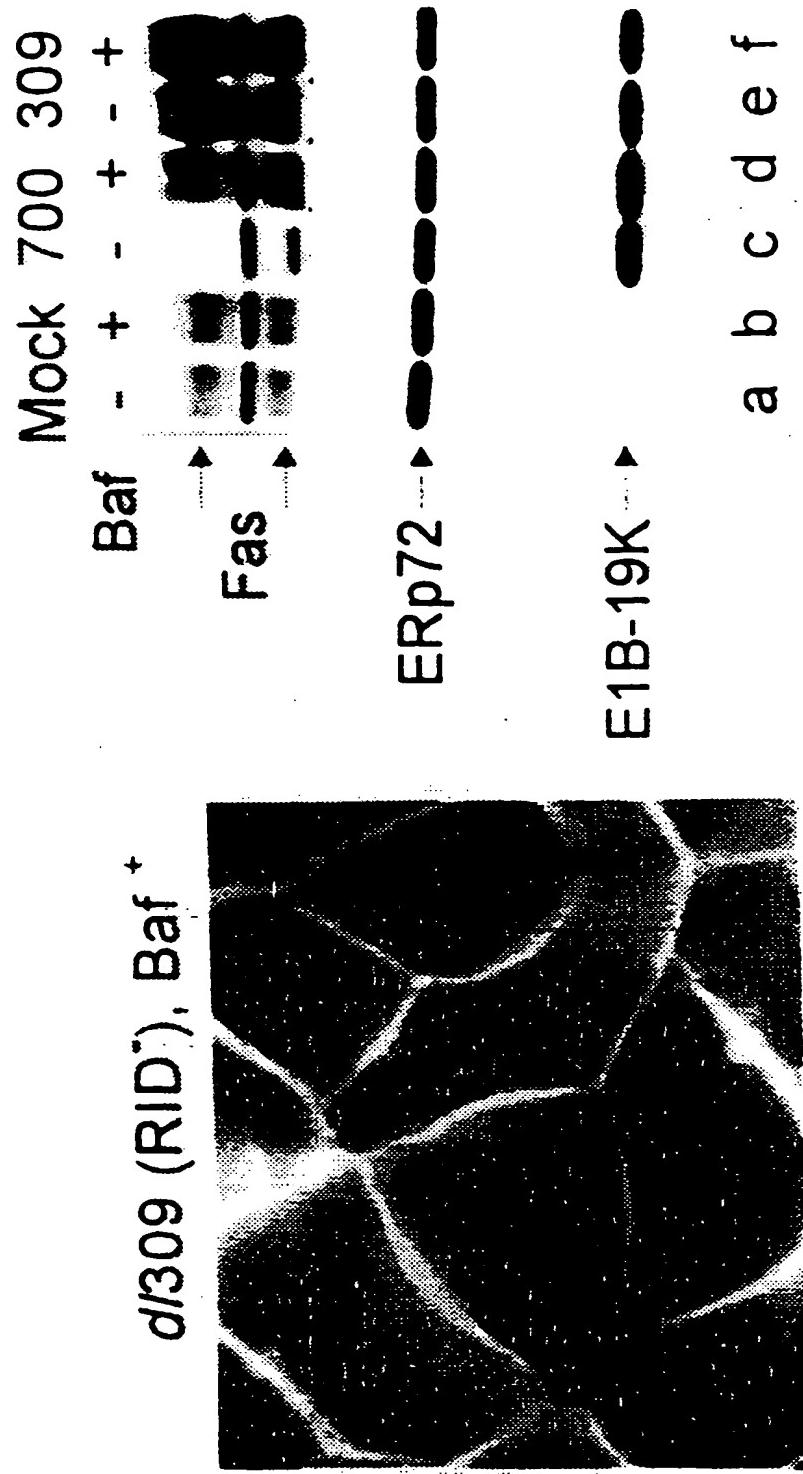


Figure 13C

Figure 13D

20/85

Mock 700 309
Baf - + - + - +
TfR - - - - -

Figure 13E

21/85

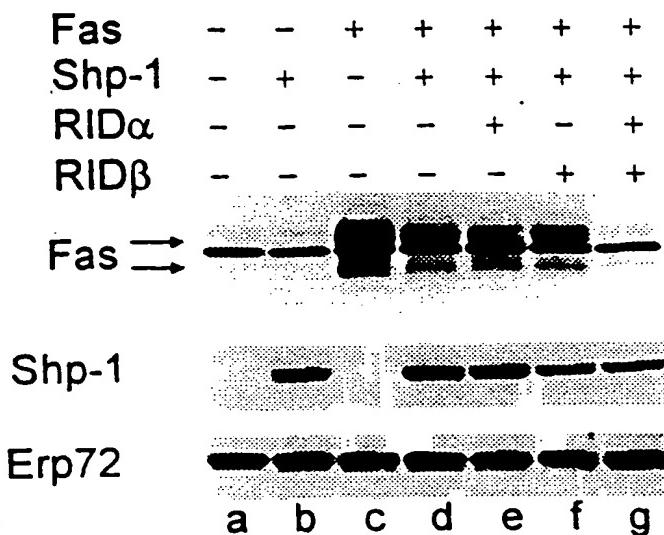


Figure 14

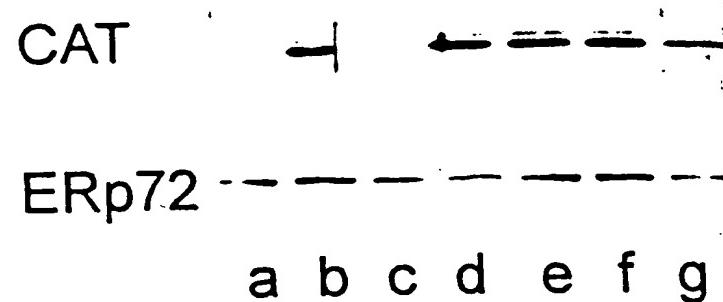
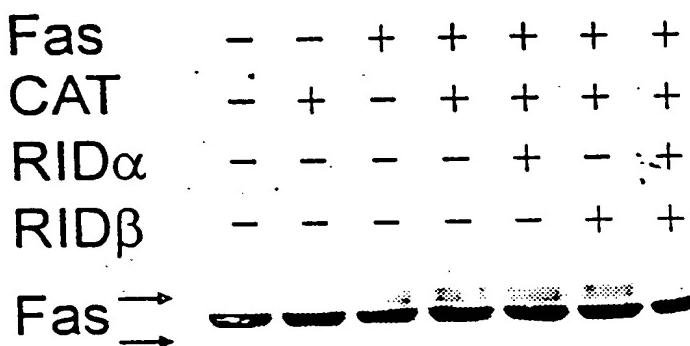
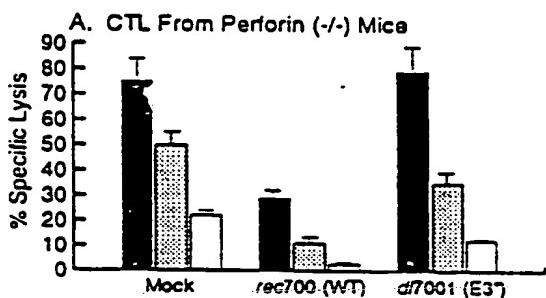
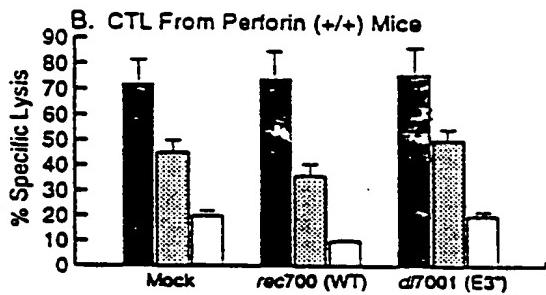
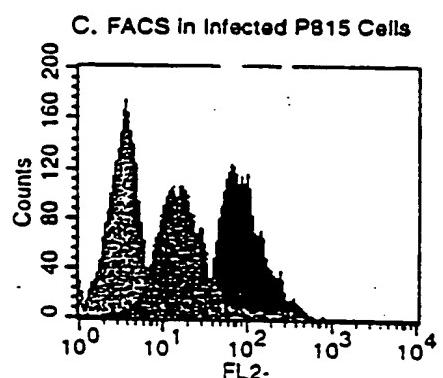


Figure 15

22/85

Figure 16A**Figure 16B****Figure 16C**

23 / 85

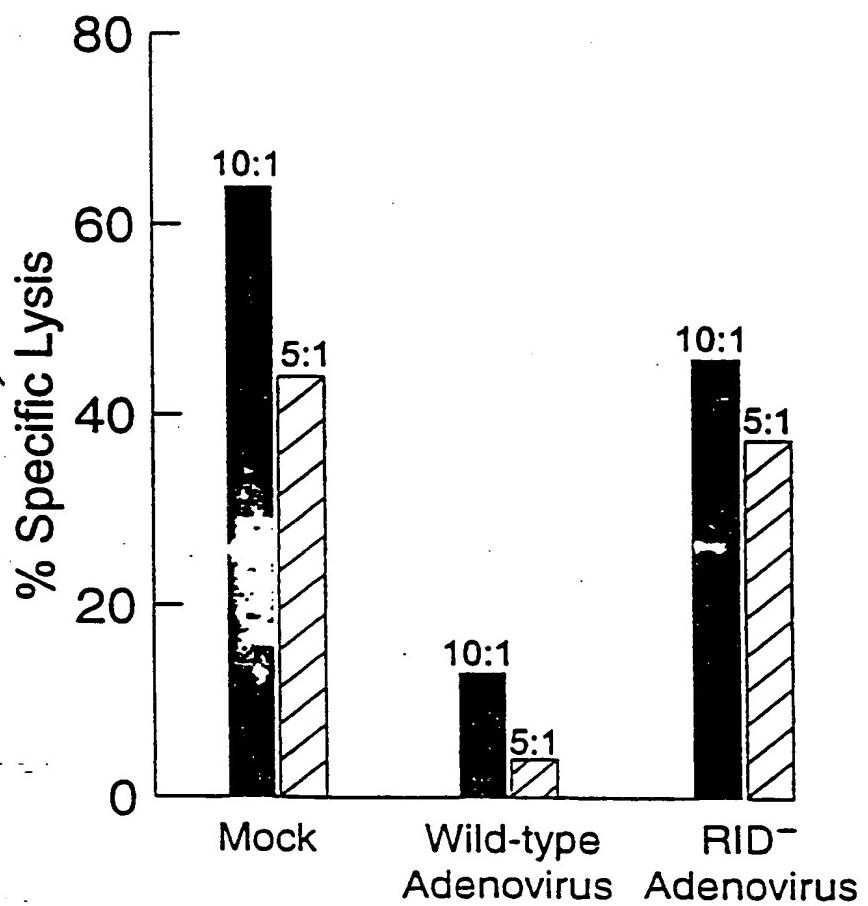
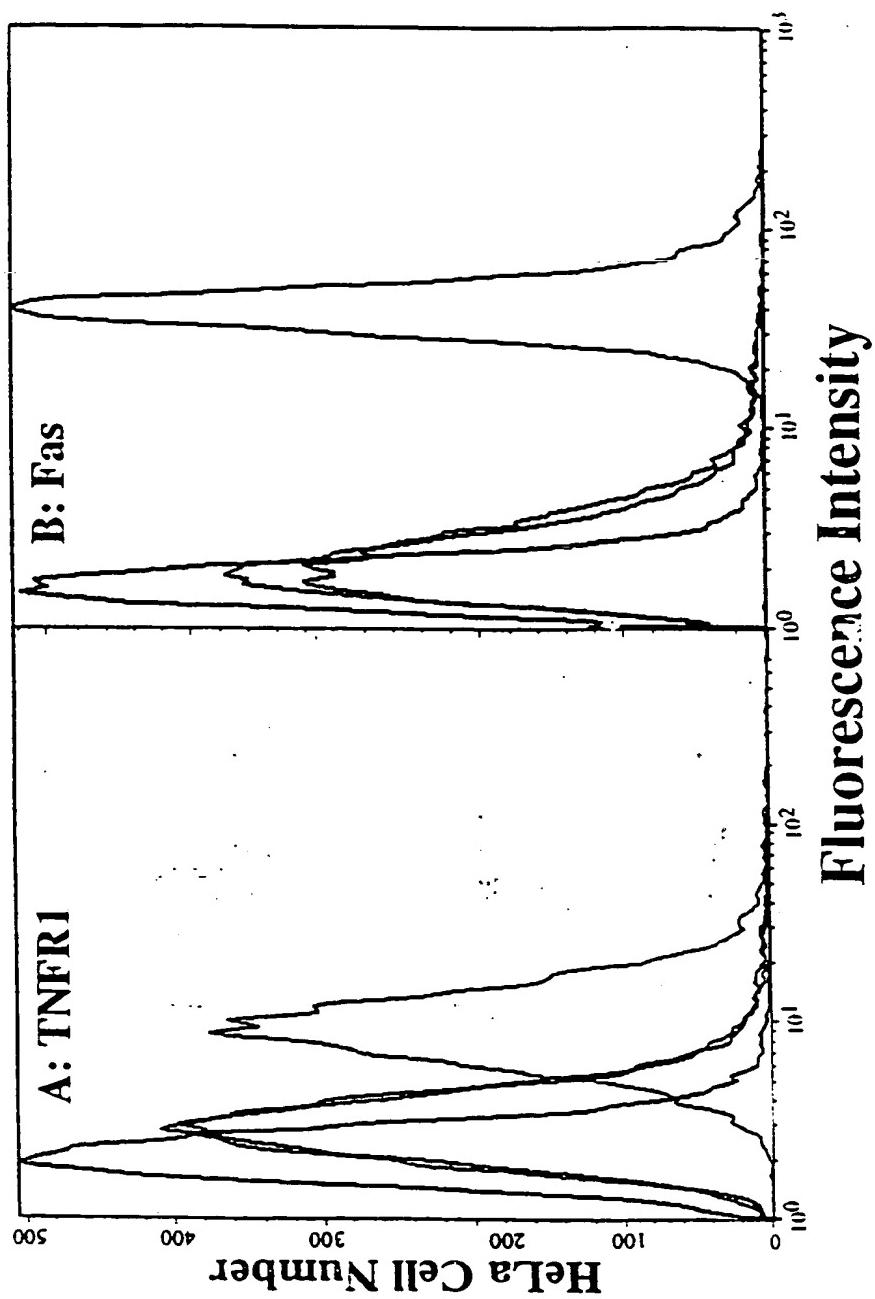


Figure 17

24 / 85



- Mock - 100%
- rec700* (Wild type) - 16%
- dl712* (\uparrow RID) - 14%
- Unstained control - 1.5%

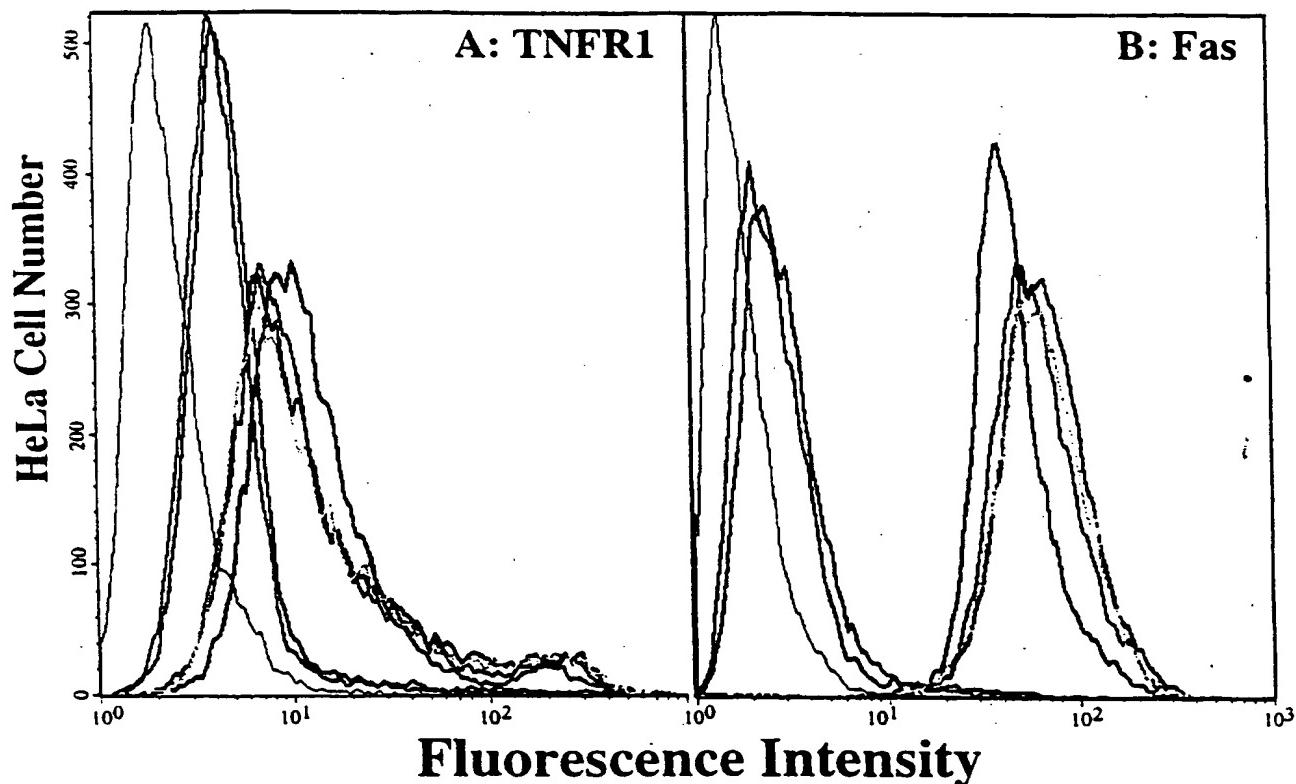
Figure 18A

Figure 18B

25/85

Figure 19A

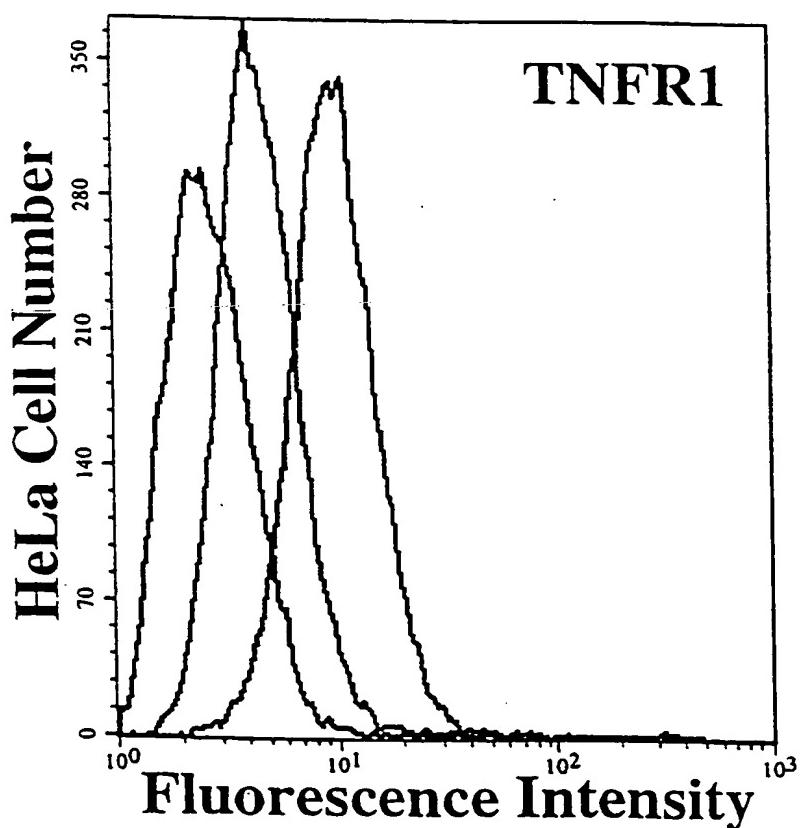
Figure 19B



Mock - 92%
rec700 (Wild type) - 29%
dl753 (RID α^-) - 85%
dl764 (RID β^-) - 84%
dl712 (\uparrow RID) - 24%
dl309 (RID $^+$) - 84%
 Unstained Control - 2%

Mock - 100%
rec700 (Wild type) - 4%
dl753 (RID α^-) - 100%
dl764 (RID β^-) - 100%
dl712 (\uparrow RID) - 2%
dl309 (RID $^+$) - 100%
 Unstained Control - 1%

26 / 85



Mock - 93%

231-10 (E3⁺ vector) 24 hr. p.i. - 35%

231-10 (E3⁺ vector) 48hr. p.i. - 11%

Figure 20

27/85

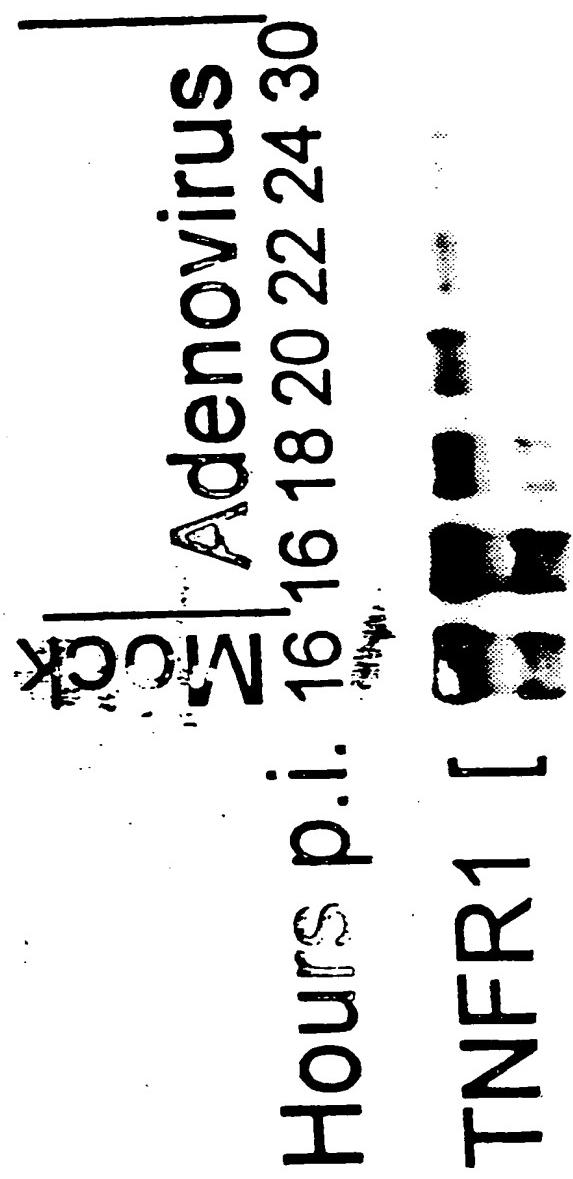


Figure 21

28/85

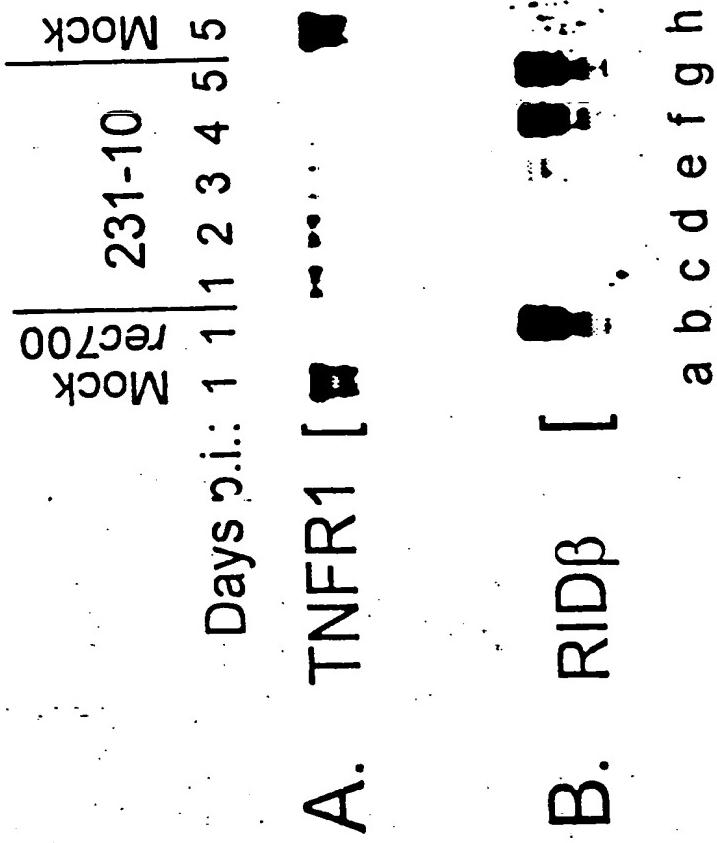


Figure 22

29/85

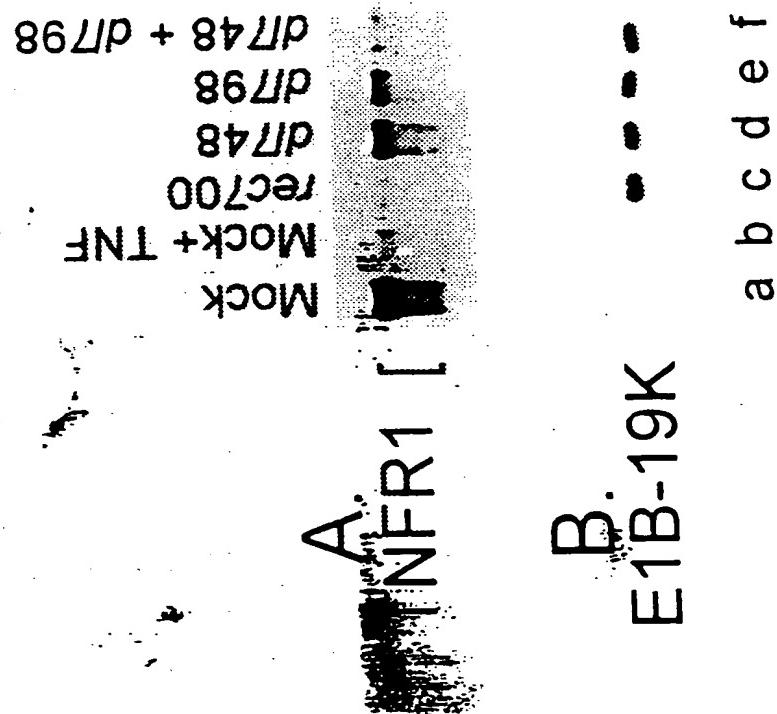


Figure 23

30/85



Figure 24

31/85



Figure 25

32/85

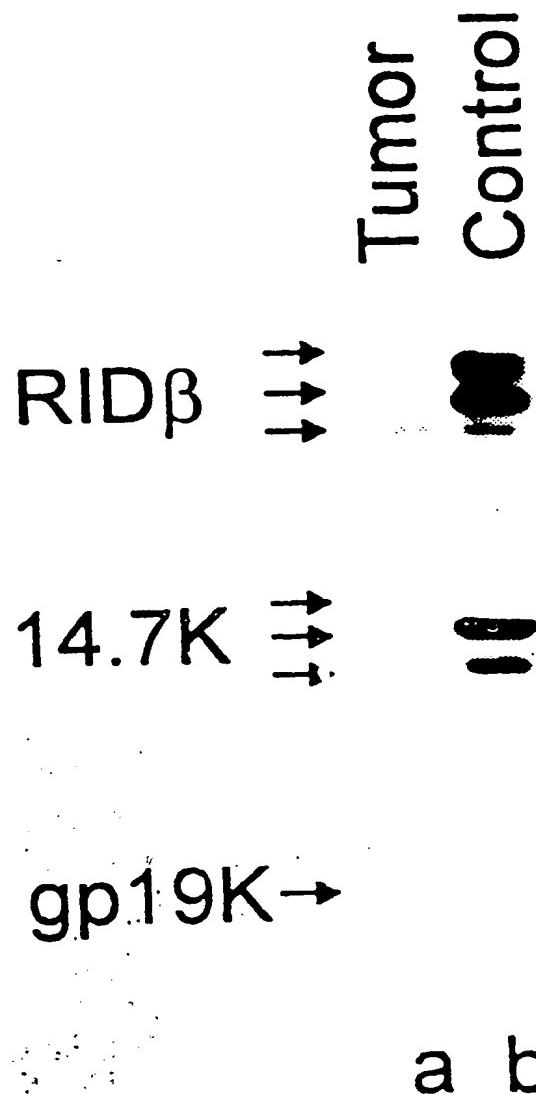


Figure 26

33 / 85

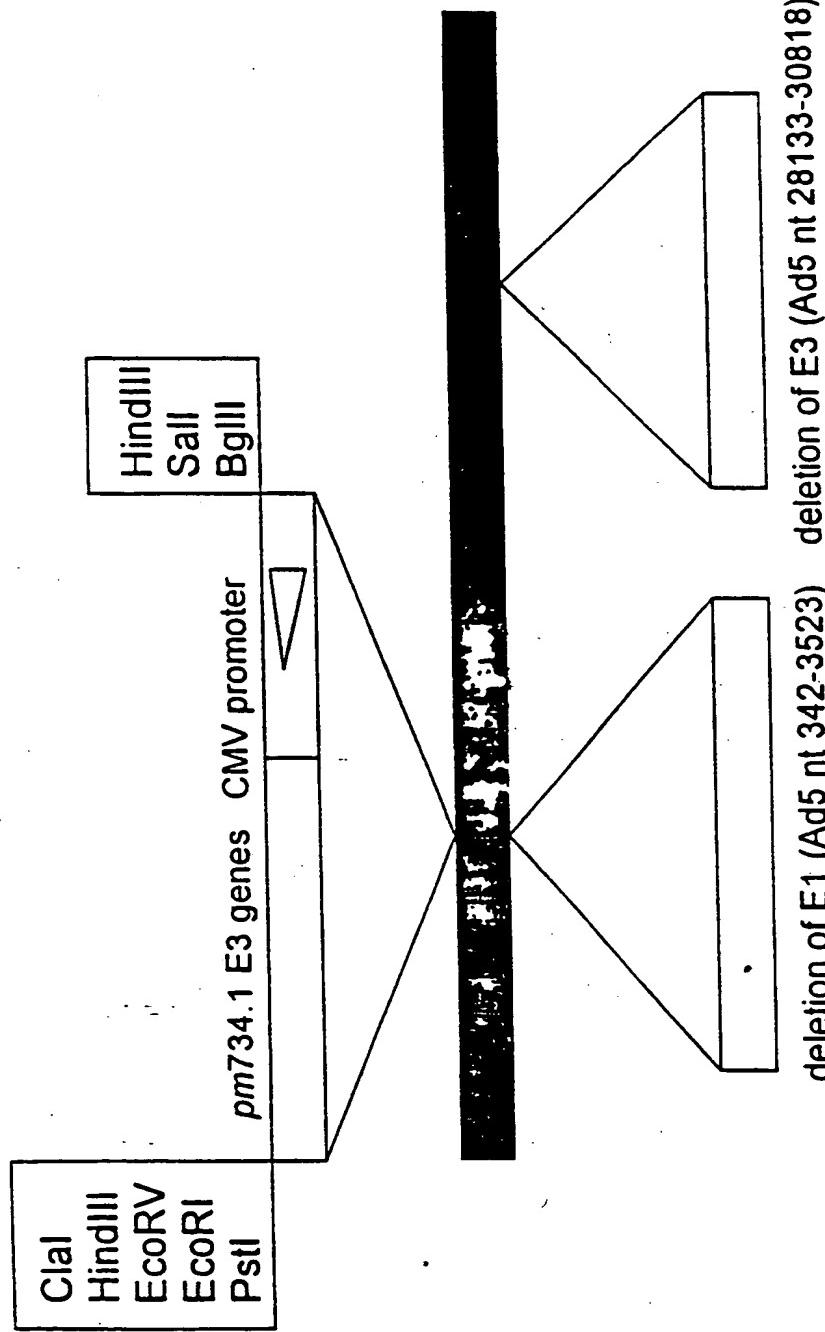


Figure 27

34 / 85

Figure 28A

35 / 85

Figure 28B

agagacataacgccttaccacggactccatgcgttacgcgttgtttggccayttccccg
 1501 SfcI SfcI PstI BspMI
 tgaatgaaggcgatgactacagtgacgaggctgcagatgaggataagggtgacaatccg 1560
 acttactttccgctactgtatgtactgtccgacgttacttccatcccactgttttagc
 EaeI
 GdIII
 SacII
 MspAII
 BsiEI |||
 BstDSI |||
 | |||
 1561 caaaggcaggtaaacgtgaaagggtggatgcaatctacttcgtatgtgagcgacccggcc 1620
 atttcgccatggacactttccaccctacgttagatgaagctacactcgctggcgccgg
 BsiHKAI Aval
 Bsp1286I SmlI
 | XbaI
 1621 aatgttagagcacgcacagaaaagcgcaacaagggtcaataatataagaactcgaggaatt 1680
 ttacatctcgcggtgttttcgcgttgtccatccatccatgtgatccatgg
 EarI ApoI
 1681 atgtctcatttaatcataactgtaaaagaagagaacatggttcagacccgttcaatctatg 1740
 tacagagtaaatttagatgacatccccccgttgcaccaaaagtctggcaggctgatata
 RleAI SfcI
 XmnI BsiHKAI HaeI HaeIV
 | Bsp1286I StuI HinI
 1741 aaaaaaaaaaaaaatgtgtgggtttagcacaatgtataggcctatagatgggggttctggcg 1800
 taaaaaaaaaaaaatgtgtgggtttagcacaatgtataggcctatagatgggggttctggcg
 Bsp24I
 HaeII |
 Tch111II |
 Eco47III |||
 | |||
 1801 tctgcgttttagcaacaaaataagccacataataataaggcaaaaaacataaagcgctat 1860
 agacgcgaaatccgttgttattcggttattatccgttttgttgcatttcgcgata
 Bsp24I
 BmrI | ApoI
 | |
 1861 gaaaaaccaccacaaggccaagtcgcggcagtcattgacaaaggcatgaacctgggttaa 1920
 ccccttgggtgttcagttcgagcgggtcagtaactgtttccgtacttgaaacccatt
 RleAI
 BsaWI BtsI BsbI
 | | |
 1921 attagggcagatgttagtcggtagcagtcgggtgtcgatagtcgtttgtggcgcat 1980
 taaaatcccgttacaatcaggccatgtcaccacaacgcgtatcaggcaaccccgctta
 HincII EcoS7I
 BsrFI BpmI BclI ApoI | BtsI
 | | | | | |
 1981 gggttagccggtaactctggagcaggcaagctgaagctgggttgcataaaatttgcgt 2040
 ccaactcggccagtttagacccgttccgcgttcgacttcgacccaaacttagttaaacgtca
 HaeII HaeII
 AlwNI BsgI BspGI RleAI
 || | | |
 2041 gcaggcgctggcagaaatcaggcgctaacgtccaggaaatgtttgtgg 2100
 cgtccgcgaccgttttagtccgcgattgcaggcccttcaaaactaaaccccaacaccc
 BspGI
 BpmI RleAI
 | | |
 2101 cttatgtttggccgtggagcatatcccacatagatgtttgtttgtgg 2160
 atattagaacggggggacctcgatagggtgtatccatccatccatccatccatccatcc
 Tch111II
 |
 2161 caagcgaaaaatcaaggcatttttttcatcaataaaactgcgtttgtttgtatcc 2220

Figure 28C

37 / 85

Figure 28D

38 / 85

2941 gcttcaggccgttaggtgtgggcgcagcaaccgggtacgcactcgtaacgtttccggcagggt
cagtcgcacatccacacccggcgctggcactgcgtgagcatgcaaggggccgtccac 3000

BseRI |
3001 aggagggtgggtgggtgggtgggttttttttgcacgggttagtgtgaagccgagaagggtgt
tcctccccaccaccaccaccacaaaaagaactgccacatcaactcggtttccaacaca 3060

BsmBI | EarI
3051 ggcaaacttacttcgtctcggtggaaacctgtgtcaaattacaaaatgaagagccgttaag
ccgttgaatgaagcagacgacccatggacaacatatacggtttacttcggcaatttc 3120

HgiEII | SexAI | BsaWI BsaXI TaqII
3121 taccaggtaaggtaacttattggcccgttgcaaaaccggagggtgggtttggc
atggccatccatgaaaaaccggggcaacacgtttggctccactccaaacgaaaccag 3180

BmrI | BanII | BsaXI | Bsp1286I |
3181 tgcttgggtggtaaaaaacgggtggcggtcacaggatggcgacaggaggcccaagttagatt
acgaaacccacccatggccacggcaagtgtccctaccgtgttctgggtcatctaa 3240

MspI | BglII | BstYI |
3241 ctaatttctgtatttattataactcagcacagagatgacaacaaagatcttgcattcc
gattaaagacataaatatgagtcgtgttctactgtttttagaactacattagg 3300

Eco0109I | Psp5II |
BstDSI | SanDI BsrBI BsrBI |
3301 agggttcaggacagttgcaaaaccacggtcagaacacagggacccgtccgtccactag
tcccaatctgtcaacgttggtccagttgtgtccctggggcgagggcgaggtgatcc 3360

HaeII | AflIII | TaqII |
3361 cagggggcgcttggtaaaactcccaatcaggctacgtgtaaagcttacccgggtggtag
gtccccccgcgaaccatggaggctttagtccgatgcacattcgagatggacccaccatc 3420

ApaI | BanII | Bsp1286I | BmgI |
BsaHI | Eco0109I | BseSI | EarI
3421 ccggacgcgtgcggggccctcgatatgtttccgggcaattaaagtaacaaaactc
ggctcgccgcacgcggccggagctatacgagaagccgttaagtttccatgttttag 3480

SacII | MspAII |
BsaWI BstDSI ||| BtsI |
3481 accggagccgcggcaaaaggacattgtggcgccggcagtggtcgaggtgttcaggcgag
tggccctggccggccgttctggaaacaccgcggccgttacccagctccacacagtccgcgtc 3540

Pfl1108I | EclI |
3541 tcgctctgccttccactggtcattcagtcgtagccgtccggccagtttttaccgcgtc
agcgagacggagaggtgaccagtaagttagcattccggcaggccgtcagaaagtggcgag 3600

EaeI | CdiI | BspCI |
3601 aaagtggaaataaactggtcgggttagtgccggggagggtccagaaaaagggttgaagta
tttcaacccatatttggccatcaccggccctccaggctttttccaaacttcat 3660

BseRI | BsaWI | BspEI | Hin4I |

Figure 28E

Figure 28F

40 / 85

4321 CGTCAATAGGGGCGTACTTGGCATATGATACTTGATGTACTGCCAAGTGGGACTTT
GCAGTTATCCCCGATGAACCGTATACTATGTAACTACATGACGGTTACCCGTCAAA 4380

TaqII
AatII |
BsaHI |

4381 ACCGTAATAGTCACCCATTGACGTCAA TGGAAAGTCCCTATTGGCCTTACTATGGAA
TGGCATTATCAGGTGGTAACTGCAGTTACCTTTCAGGGATAACCGCAATGATAACCTT 4440

AatII
BsaHI | BglI
|

4441 CATACTCATTATTGACGTCAATGGGGGGGGTCCTTGGGGTCACCCAGGGGGCAT
GTATGCAGTAATAACTGCAGTTACCCGCCCCAGCAACCGCAAGTGGTCCGGGGTA 4500

4501 TTACCGTAAGTTATGTAACCGGAACCTCCATATATGGCTATGAACTAATGACCCGTAA
AATGGCATTCAATACATTGCGCTTGGAGTATATACCGATACTTGATTACTGGGGCATT 4560

AflIII
VspI SpeI | MluI
| HincII |

4561 TTGATTACTATTAATAACTAGTCATAATCAATGTCACCGGTATATCTGGCCGTACAT
AACTAATGATAATTATTGATCAGTTATTAGTTACAGTTGCCATATAGACCGGCATGTA 4620

HincII
NruI Bpu10I Bsp24I AccI
| HindIII SalI |||

4621 CGCGAAGCAGCGAAAACGCCAACCTAACCGAGATTCTTCATGCAATTcaagcttgcg
GGCCTTCGTCGGTTTGGGATTGGGATTCGCTAAGAAGTACGTTAAGttcgaaacagc 4680

BglII AflII Bsp24I
BstYI SmlI | |

4681 acagatcttggcggtggcttaagggtggaaaagaatataagggtggggctttatgtag
tgtctagaacccgcaccgaattccccaccccttttttatataattccaccccccagaatacatt 4740

BsiHKAI
Bsp1286I |

4741 ctttgcattctgtttcgagcagccgcgcgcacatgaggcaccaactcgcttgatggaaac
aaaacatagacaaaacgtcgccggcgccgtactcgctggctttagcaactacccctcg 4800

BstDSI NcoI
BanII Bsp1286I StyI |
BsiHKAI | NspI |
Bsp1286I | SacI | SphI | BpmI |

4801 attgtgagctcatatggacaacgcgcacatggccggggatggccggggcggtcagaatgtg
taacactcgagataaaactgttgcgcgtacggggatccggcccccacgcagtcctcacac 4860

Pfl1108I
BanII BsaI |
Bsp1286I Bsp24I | |
| |

4861 atgggcctcagcactgtggcgccccgtctggccggaaactctactacccgtacccctac
tacccgaggctgtactaccagcgggcaggacggcggtttagatgtggaaactggatg 4920

PstI
Tch11II Eco57II SfcI
Hin4II | SfcI | | AlwNI
MmeI || Bsp24I | | EcII | MspAII|PstI
| | | | | | | |

4921 gagaccgtgtggaaacgcgcgtggagactgcaggctccggccggcttgcggctgtca
ctctggcacagacccgtggcaaccttgcgtcgaggccggccgaaactcgccgacgt 4980

SacII BanII
MspAII Bsp1286I BstAPI
BstDSI || Bpu10I | BtsI |

4981 gccaccgcggcgccgttgcgtactgacttgcgtccctggagccggccgttgcgtca
cggtggcgccctaaacactgactgaaacgaaaggactcgccgaaacgttcgtcacgt 5040

BsgI

Figure 28G

41 / 85

Figure 28H

42 / 85

ApaI
 BanII
 Bsp1286I
 BstDSI
 BmgI |
 NsiI BstXI MsI BseSI |
 BsrDI |
 cattcgtccataatgatggcaatggcccacgggcggcggctggcgaagatatccg 5761
 gtaaggcaggattactaccgggttacccgggtggccggccggacccegtttctataaaagac 5820
 MsI HaeI
 ggatcaactaacgtcatagttgtgtccaggatgagatcgcataggccat:aaaaag 5821
 cctagtgtatttcacacaaggcttactcttagcgtatccggtaaaaatgtttc 5880
 EcII BanI DrdII
 cgccggccggagggtgccagactgcggataatggccatccggccaggggcgtagtt 5881
 gccccggccctccacggcttgacgcccatttaccaaggtagggccggccccccatcaat 5940
 AccI
 ccctcacagatccgtatccacgcggatggggatcatgttacctgc 5941
 gggaggtgtttaaacgtaaagggtgcgaaacctcaagtttaccccttagtacagatggacg 6000
 BspMI BspMI
 MspAI Pvull
 gggcgatgaagaaaacggttccgggttagggagatcagctggaaagaaacgggttc 6001
 ccccgctacttctttgtccaaaggccccatccccctttagtcgacccttttgtccaag 6060
 ApaI
 BanII
 Bsp1286I
 MspAI BsrFI BmgI |
 Pvull HgelIII BseSI |
 ctgagcagctgcgacttaccgcagccgtggccggtaatcacaccttaccgggtgc 6061
 gacttgcgacgcgtaatggcgccggccacccggcattttgtgtggataatggcccacg 6120
 MspAI Pvull
 PstII
 AceIII SfcI || Bpu10I
 aactggtagttaaagagagctgcagctgccgtatccctgagcagggggccacccgtta 6121
 ttgaccatcaatttccgcacgtcgacggcagcaggactcgccccccgggtgaagcaac 6180
 NspI NspI EciI HaeII
 agcatgtccctgactcgcatgtttccctgaccaaataccggccagaaggcgccggcc 6181
 tcgtacaggactgagcgtacaaaaggactggtttaggcgtttccgcgagcggccgg 6240
 BstAPI BsaI EciI
 agcgatagcgttttgcagaaaggaaagtttccaaacgggttagacccgtccggccgt 6241
 tcgctatcgccaaacgtttttcgttttcaaaaagggtgcacacttggcagccggcat 6300
 RleAI
 NspI TaqII AcelIII
 SphI Tth111I BstEII AarIII
 ggcatgttttgcgcgttgcaccaaggcgtttccaggcggtccacagctggcacctgc 6301
 ccgtacgaaaactcgcaactggttcgtaaggctcgccagggtgcgagccactggacg 6360
 BspMI BseRI
 cttacggcatctcgatccagcatatccctcggtccgcgggtggggcggcttcgtgt 6361
 agatgccgttaggtcgatagaggagcaagcggccaaacccggccaaagcgcaca 6420
 BspGI
 BsiHKAI BstDSI EcoO109I
 Bsp1286I BcgI | PspSII
 acggcagtagtcgggtgcgtccagacggccagggtcgatccacggccggcagg 6421
 F
 6480

Figure 28I

43 / 85

Figure 28J

44/85

NspI
BbsI
PshAI
AflIII
BspLU₁II
EarI
7081 ctcgctccagggtgtgaagacacatgtcgccctttcggtcatcaaggaagggtattggtt
gagcgaggtcccacacttctgttacagcggagaagccgtagttccactaacc
BsaAI
PmlI
HaeI
EcoS7I
7141 tggtaggtgtaggccacgtgaccgggtgttcttgaagggggccataaaaagggggtgggg
acatcccacatccgggtcactggcccacaaggacttcccccgatatttccccac
PflMI
MspAI
PvuII
TatI
EarI
7201 cgccggccgtccctacttctccgcacgtcgctgttgcgaggggccagctgtgggtgagt
gccaaggcaggagtggagaaaggcgtagcgacacagcgtcccggtcgacaacccactca
actccccctgtaaaaaggccgtactgactttctgcgctaagatgtccagttttccaaaaacgagg
tggggagacttttgcggccgtactgaagacgcgttttaacagtcaaaagggttttgc
SacII
MspAI
BseRI
BstDSI
EaeI
GdII
7261 agggatttgatattcacctggcccggtgatgccttgggggtggccgatccatctgg
tccctaaactataagtggaccggccactacggaaacctccaccggcgtaggttagacca
HindIII
MmeI
7321 cagaaaaagacaatccccctgttgcataagctggggccaaacgacccgttagggcggtgg
gtccccctgttagaaaaacaacagttcgaaaccacccgttgcggcatccccgcaacc
BssHII
BsiEI
PvuI
EaeI
GdII
NrI
StyI
7381 acagcaacttggcgatggagcgccagggtttttgtcgatcgatccggcgccgttgg
tgtcggttgcggccgttccaaacccaaaaacagcgcttagccgcgaggaaacc
BsaAI
BssHII
7441 ccgcgcgttttagctgcacgtattcgccgcgcacgcaccggccatccggaaagacgg
tgtcggttgcggccgttccaaacccaaaaacagcgcttagccgcgaggaaacc
BsiHKAI
Bsp1286I
BseSI
ApaLI
BstAPI
DraI
Bsp1286I
SexAI
BmgI
BseSI
BanII
7501 SacII
MspAI
BstDSI
Tth11I
HincII
tgcgttgcgtggccaccagggtgcacgcgcacccgggttgtgcagggtgacaagg
ggcgcttgcgtggccaccagggtgcacgcgcacccgggttgtgcagggtgacaagg
7561 tgcgttgcgtggccaccagggtgcacgcgcacccgggttgtgcagggtgacaagg
acgcgagcaggccgtggccacgtgcgcgggtggcgccaaacacgtccactgttcc
BsiEI
EaeI
EagI
GdII
NoI
BsgI
HaeII
BspGI
7621 BsmBI
BstDSI
7681 gcgagcagaatggcggttaggggtctagctgcgttgcgtccgggggtctgcgtcc
cgctcggttccaccgcacccaggatcgacgcagacgcaggccccagacgcagg
7681 7740

Figure 28K

45 / 85

Figure 28L

8461 ----- 8520
 tccacagggacttggtaactgaaacctccatgaccataaacttcagtcacagcagcgtaggg
 cctgctcccagagcaaaaagtccgtgcgtttttggaaacgcggatttggcagggcgaagg
 8521 ----- 8580
 ggacgagggtttcggtttttaggcacgcgaaaaaccccttgcgcctaaaccggccccgccttc
 EcoO109I
 EarI | PspSII
 | SanDI
 tgacatcggtgaagagtatcttccgcgcgaggcataaaagttgcgttgtatgcggaaagg
 8581 ----- 8640
 actgttagcaacttcatagaaaaggcgccgtttttcaacgcacactacgccttc
 BsiHKAI
 Bani | Bsp1286I
 |
 gtcccccaccccgaaacggttgttaattttacctggggggcgagcacgatccgcctaaagg
 8641 ----- 8700
 cagggccgtggaggccctgccaacaattaaatggacccgcgcgttgttagagcaggttcg
 RleAI
 |
 cgttgatgtttgtggcccaaaatgtaaatgttccaaagaagcgcggatgccttgatgaaag
 8701 ----- 8760
 gcaactacaacacccgggttttacatttcaaggttttcgccctacgggaaactaccc
 BsiHKAI
 BanII | Bsp1286I
 BsiHKAI | Bsp1286I
 Bsp1286I | Bpu1102I
 Pfll108I | SacI EarI |
 Eco57I | AcelII | SapI |
 gcaatttttaatgttctcgtaggttagctttcaggggagctgagccctgtgtctgaaa
 8761 ----- 8820
 cgttaaaaaatttcaaggagcatccactcgagaatgtttttcgactcgccacgagactt
 BmrI
 Apal
 BanIII
 Bsp1286II
 MmeI|||
 BmgI|||
 BseSII|||
 EcoO109I |||||
 |||
 gggcccaagttcgcaagatgaggggttggaaagcgaatgagttccacaggtcacgggca
 8821 ----- 8890
 cccggggccagacgttttactcccaaccccttcgcgtttttactcgagggtttccatgc
 EcoO109I
 PspSII
 Sse8647I
 BspMI AarI NruI | HaeI
 | | | MscI
 | | | EaeI |
 tttagcatttgcagggtggtcgcgaaaggcttcaaactggcgacccatggccatttttcg
 8881 ----- 8940
 aatcgtaaacgtccaccaggcgcttttccaggatttgaccgtggataccggtaaaaaagac
 MspAII StyI
 | |
 ggggtatgcagtcagaaggtaagcgggtttttttcccaagggtttcgccgg
 8941 ----- 9000
 cccactacgtcatttccatttgcggcaaaagggtcgccaggtagttccaaaggccc
 BsaI EcII RcaI AcelII
 | | | |
 cttaggtttcgccggcagtcactagaggcttcatctccgcgaacttcatgaccaggatga
 9001 ----- 9060
 gatccagacgcgcggcgttgcgtatcccgaggtagaggcggtttgaagtactggtcgtact
 BstAPI
 Bsp1286I |
 BssSI |
 BmgI|||
 BseSII | | EcoO109I | Pfll108I
 ||| | | | | BsaI | BsmBI
 agggcacgagctttcccaaggccccatccaaggatataaggcttccacatcgtaggtga
 9061 ----- 9120
 tcccgtagttcgacgaagggtttccggggtaggttcatatccagagatgtacact
 BsaXI
 AloI
 BsiEI BstYII
 Pvul UbaII MunI
 | | | | |
 caaagagacgcgggtgcgaggatgcgagccatcgggaaagaactggatcccgccacc

Figure 28M

9121 -----+ gttttccctcgccggccacgccttacgcctcggttagcccccttcgtgacccttagggccggcgg 9180
 PflMI BseRI BssSI
 | |
 9181 aattggaggagtggctatgtggtaaaagtagaagtccctgcgacggccgaacact | 9240
 ttaacctccaccataactacaccatcatcttccaggacgtgcccggcttgcga
 BsiHKAI
 Bsp1286I
 ScaI BsgII
 TatI BseSII
 FspI ApaLI !!! BsrGI
 | | MspAII | ||| TatI
 9241 cgtgtggctttgtaaaaacgtgcgcagactggcagcggtgacacggctgtacatccc | 9300
 gcacgaccgaaaacatccccgtacgcgtcatgaccgtcgccacgtgcccggacatgttagga
 BssSI BanII
 EcoNI HincII BsiEI ApoI Bsp1286I
 | | | |
 9301 gcacgagggttgcacctgacgaccgcgcacaaggaaaggcagatggaaatttgagccccctcgc | 9360
 cgtgtcccaactggactgtggcgtgtccccctcgatcacccctaaactcgccccggagcg
 AvAI
 BbsI Tth111I
 | |
 9361 ctggcggtttggctggcttctacttggctgtttgtccccctgaccgtctggccgt | 9420
 gaccgcggcaaccgaccaccagaagatgaagccgacgaaacaggactggcagaccgacga
 BsaXI BanII MmeI
 | | |
 9421 cgagggggagtacggtggtcgaccacccacgcccgcgcgagcccaaagtccagatgtccg | 9480
 getccccctcaatgccacctagccctggctggcggcgcgtccccctcaggatcacaggc
 BstDSI NcoI
 BsiEI AceIII Styl PflMI
 | | | |
 9481 cgcgcggcggtcgagcttgcataacacatcgccgatggagctgtccatggcttgg | 9540
 ggcgcggccagccctgaactactgttgcgcgttccatggatggcggatccatggccatgg
 BsaHI SacII
 | |
 MspAII |
 BstDSI | |
 BanII | |
 BsiHKAI | |
 Bsp1286I | |
 SacI | | BpmI BspMI SacI | SbfI
 | | | |
 9541 gtcggcggtcgagttcaggcaggccggggagcttgcagggttacctcgcatagacgggtca | 9600
 cgagggcgccgcagtcaggccggggccatggatggatggatggatggatggatggatgg
 XcmI
 AlwNII
 BstYI PflMI BsaHI
 | | |
 9601 gggcgccggctagatccagggtataccatccatggatggatggatggatggatggatgg | 9660
 cccgcggcccgatctaggatccatggatggatggatggatggatggatggatggatgg
 SacII KpnI
 MspAII |
 BstDSI | |
 Hin4I BaeII | |
 | | | |
 9661 tggcttgcacggggccgcattcccgccggcgactacggatcccgccggggccggatgg | 9720
 accgaaatggatcccgccgtatggatggatggatggatggatggatggatggatgg
 SacII NsiI
 MspAII |
 BstDSI | |
 StyI HaeIV Hin4I |
 | | | |
 9721 cgcgggggggtccccctggatgtgcattaaaagcggtgacgcggccgagccccccggagg | 9780
 Fig

Figure 28N

Figure 280

49 / 85

Figure 28P

catcaccagaacgtactcgaaagatggccgtgaagaagaagaggaga-ayyacg
 HaeII
 BsaHI
 NarI
 BstAPI EaeI GdII SanI SalI
 atctttgcataatcgctggggggggggaggtttggccgtatgtggccccccttcc
 tagagaacgtagatagcgacgcccccccccaaacccggcatccaccggggagaagg 11220
 MsI I EcoRI
 tcccatgcgtgtgacccccaagccccatcggctgaagcagggtaggtcgccgacaac 11280
 agggtaacgcacactggggttcggggagtagccgacttcgtcccgatccagccctgttgt
 BspMI
 EccNI HaeI AhI
 MslI AarI AccI Hin4I
 BsgI HaeI AarI AccI Hin4I
 ggcggccggtaatggcctgtgcacccgggtggtagactggaaagtcatccatgtcc 11340
 cgcgagccgattataccggacgacgtggacgactccatctgacccatccatgtacag
 HaeI
 MscI
 BsbI EaeI BsgI
 cacaaggcggtggtatgcggccgtgtgatgggtgtaaatgcggccatcaaaccggcca 11400
 gtgtttcgccaccatcacgcgggcacaactaccacatccacgtcaaccgggtattgggt
 BsmBI
 BanII BsmKAI Avai
 HincII BstEII Bsp1286I SmI
 HpaI SacI XbaI
 gttaaacggcttggtgacccgggtgcgagactcggtgtacctgaaatgcggagtaaccct 11460
 caattggccagaccactggggacgcttcgagccacatggacttgcgtcatccggga
 BsaAI AlwNI
 SnaBI SexAI PflMI BciVI
 cgagttcaatacgttagtgcgttgcacgtccgcacccgggtactggatcccaaaaaatg 11520
 gtcgtttatgcatacgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcac
 EaeI BanII BglIII
 GdII Bsp1286I BstYI
 cggcggcggctggcggttagggggccagcgttagggggccggccggccggccggccggcc 11580
 gccggccggccggccatccccgggtcgatccacccacggggccggccggccggccggcc
 EcoRV MmeI BspGI MslI NgoAIV BsrFI
 ttccaaacataaggcgatataccgttagatgtacccgtggacatccatggatggccggcc 11640
 aagggtgtatccgtactataggcatccatggacccgttaggtccactacggccggcc
 BssHII MspAII FspI BstAPI
 Ggtgggtggaggcgccggaaaaggcgccggacccgggtccagatgttgcgtggccggcc 11700
 ccaccaccccccggccgttccatggccggccatccatggccggccggccggccggcc
 BstDSI NcoI StyI BsrFI BssHII BsrFI
 BsmKAI EaeI BssHII GdII BglI HincII XbaI
 Bsp1286I Bsp1286I Bsp1286I Bsp1286I Bsp1286I Bsp1286I
 gtgtccatggtggggacgttccggccggccatggccggccatggccggccatggccggcc 11760
 cacggggcaccagccgtcgagaccggccatccggccggccatggccggccatggccggcc
 Bsp1286I BmgI Eari
 BsaXI BseSI BstDSI ApoI
 cgtgcggaaaaggagggccgtaaagcgggactttccgtgggtggataaattcgcaa 11820
 gcacgtttcccttcggacactcgccccggagaaggcaccagaccatccatggccggcc

Figure 28Q

51/85

Figure 28R

12361 cgaaccccccggcggccggccggactacctggacttggaggagggcgaggcccggc
gcttggggcgccggccggccgtatggacctgaacctccccgtccccggaccg 12420

BanI BsrBI StyI MspAII AfI II BsgI
HaeII Bpu10I KpnI PvUII MluI
gccccgttagggcgcccttcctccgtacggtaaccaagggtgcagctgaagcgtgatacgc
cgccgatccctcgccggagaggactcgccatgggtccacgtcgacttcgcactatgcgc 12480

SacII MspAII BstDSI
BsaAI SunI BsiEI BanI
Eco57I AlwNI NruI Bsp1286I AvaI
cgaggcgtagtgcgtccggcagaacctttcgccgacccggcggagggagaggagccgagga
actccgcacgcacggcgccgtttggacaaagcgtggcgcccttcctccggccccc 12540

HaeIV Hin4I A10I BsrBI
BseRI BseRI AceIII HaeI NruI
gatgcgggatcgaaagtccacgcaggcgccgagctgcggcatggctgaatcgcgagcg
ctacgccttagcttcaggtgcgtcccgccgtcgacgcgtaccggacctagcgccgc 12600

BanII Bsp1286I BssHII
BseRI BssHII
gttgctgcccggaggactttgagccgcacggcgaaaccggatttagtcccgccgcgc
caacgacgcgtccctctgtaaaactcggtcgccgtggccctaattcaggcgccgc 12660

BsiEI EaeI Eagi GdIII
BsaAI PmlI BstEII DrdII
AflIII NotI SexAI acacgtggcgccgcgcacccgttaaccgcatacgaggcagacggtaaccaggagattaa
tgtgcaccgcggcggtggaccattggcgatgtccgtctggccacttggcccttaatt 12720

SunI BsaAI BssHII SfcI
HindIII PmlI BssHII BpmI
ctttcaaaaaagcttaacaaccacgtcgctacgtttgtggcgccgaggagggtggctat
gaaaggtttttcgaaattgttggcgacgcgtcgacaccgcgtccaccgcata 12780

RleAI BseRI NsiI BssHII BpmI
aggactgtatgcattgtggactttgttaaggcgccgtggaycaaaaacccaaatagaagcc
tcctgaccacgttagacaccctgaaacattcgccgacccgtttgggttatacggttgg 12840

MspAII BsrBI PvUII BsmI BsgI
BsrBI BsmI BsgI
gctcatggcgccagctgtttatagtgcagcacaggcaggacaacggaggccatccaggaa
cgagtaccgcgtcgacaaaggatattacacgtgtcgccctgttgcgtccgttgg 12900

BanII Bsp1286I AvI MspAII BsaBI SfcI
Bsp1286I AvI MspAII BsaBI SfcI
tgcgtgtctaaacatagtagagccccggggccgtggctgtcgatccgtatggataaaacatcc
acgcgacgatccgtatcatctcggtcccgccgaccgcacgcgtaaactatccgttagga 12960

PstI MsI SmlI Bce83I EaeI GdIII
Bce83I EaeI GdIII
gcagagcatgtggcgaggagcgcgtggctgtcgatccgtatggccatcaa
cgctcgatccaccacgtccgtcgacactcgaccgcgttccaccggcggtatcc 13020

Figure 28S

53 / 85

Figure 28T

Figure 28U

14341 caggaggacacggggcagccctggaggcaacccaaactacccgtgaccaccggggcag
 gtcctccctgtgcggacccggatggatggacgtggggcccgcc 14400
 BstYI DraI BsrRI BsaAI
 | | | |
 14401 aagatccccctcgttcacagttaaacagcgaggaggcgcatccgtacgtcag
 ttcttaggggagcaacgtgtcaaatttgcgtccctcgctaaaacgcgtacgtac 14460
 BspGI HaeII XcmI
 BsgI | | |
 14461 cagagcgtgaggccctaacccgtacgcgcacggggtaacgcggcgtggacatg
 gtcgtcgaactcgaaattggactacgcgtgcggccatgcgaccgtac 14520
 BsiEI EaeI EagI GdII
 BssHII NspI BsrFI
 | | | |
 14521 accgcgcgaacatggAACCGGGcatgtatgcctaaacccggcgatcaaccgccta
 tggcgccgttgtacattggccgtacatacggagttggccggaaatagtggcgat 14580
 BsiEI EaeI EagI GdII NotI Avai
 | | | | |
 14581 atggactacttgcacgcgcggccggatgtaaaccccgagtatccatggccatcttgc
 tacctgtgaacgtagegcgcggggcacttgggctcataaaagtgggttacggtagaaac 14640
 Bsp1286I Avai BmgII BseSII BanI
 | | | |
 14641 aacccgcactggctaccgcggccggatgtggggatccggatgggggt
 ttggcgccgtgaccgtggcgccggaccaaagatgtggccctaaagctccacgggtccca 14700
 AlwNI BstAPI
 |
 14701 aacgtggattccctgggacgacatacgtggccggatccggatggggatccggatgg
 ttgtacctaaggagaccctgttatctgttgtccgcacaaaaggggcgatgggttccgg 14760
 HaeII HindIII
 | |
 14761 ctgttagatgtcaacagcgccgagcaggcaggcgatggcgatggaaaggaaaggcc
 gacgtatcaacgttgtcgccgttgtccgtccgcgcacgtttccgtcgaaaggcg 14820
 SacII MspAII
 HaeI Tth111II HaeII BstDSI
 | | | |
 14821 aggccaaggcgttgtccgtatccggatccggatccggatccggatccggatccgg
 tccggatccggatccggatccggatccggatccggatccggatccggatccggatccgg 14880
 HindIII Hin4I BsaI BglI
 | | | |
 14881 tttcaaggctgtatagggttccgtaccaggcactccgcaccacccggcgccgtgg
 aaaggctcgaaactatcccagagaatggtcgtgagcgtggcgccggacgacccg 14940
 BseRI PstI BspMI
 | | |
 14941 gaggaggatccaaacaaactcgatgtgcggccgcggccggaaaaacccgtccgg
 ctccctctatggatgttgagcgtccgcgtccgcgtttttggacggaggc 15000
 BbsI SmaI
 | |
 15001 gcattttccaaacaacggatagagccatgtggacaagatgagtagatggaaagacgtac
 cgtaaagggttggccctatctctggatccacgttttactcatccatccctcgatg 15060

Figure 28V

56 / 85

Figure 28W

57 / 85

PinAI | BmrI | NspI |
 acgaccgggtcgactggggggcgacccgaaaaccatccctgcataccaacatgccaaatg
 15661 tgcgtggccagcgtgaccccgccgtggacttttggtaggacgtatggttgtacggtttac 15720
 XmnI |
 tgaacgaggcatgtttaccaataagstttaaaggcgccgggtatggcgccgttgccttgccta
 15721 acttgtctcaagtacaaatggttattcaaattccgcggccactaccacagcgcaacggat 15780
 AceIII | TaqII | AvaI |
 ctaaggacaatcagggtggagctgaaatacgagtggtggagttcacgtgcccggaggca
 15781 gattccctgttagtcacccgtactttatgtctaccacccatcaagtgccacgggtccccgt 15840
 BsaI | BsiEII | BsiHKAI |
 actactccgagaccatgaccatagacccatgaaacaacgcgtatgtggagcactacttga
 15841 tcatgaggctctggtagtggatctggatacttggctgcttagcaccctgtatgaact 15900
 Eco57I |
 aagtgggcagacagaacacggggttctggaaacgcacatggggtaaaggcttgcacacccgca
 15901 ttccacccgtctgtcttgcggccaaagaccccttgcgttagccccatccaaactgtgggcgt 15960
 BmrI | Tth11I |
 acettcagactggggtttgcaccccgactggcttgcgtatgcgtgggtatataacaaacg
 15961 tgaagectgacccaaactggggcaatgtgaccagaacagtgacggacccatataatgtttgc 16020
 aagecccccacatccagacatcatgggtgccaggatgcggggggacttacccacacggc
 16021 ttcggaaaggtaggtctgttagaaaaacgacggccctacgcggccaccctgaagtgggtgtcg 16080
 RleAI |
 TaqII |
 Bpu10I |
 Gctttagcaacttggggatccgcacggcaaccccttccaggagggtttaggatca
 16081 cggactcgatgtaccaacccgtggccgttggaaaggccccccggaaatccatgt 16140
 Pf11108I | MmeI | BpmI | BsaHI | BglI |
 cctacatgtatctggagggtggtaacatcccgacccgtggatgtggacgcctaccagg
 16141 ggatgtacttagaccccccacccatgtcaaggcggtgacaacccatgcggatggccc 16200
 BtsI |
 cgagcttggaaaatgacaccgaacacaggcgccgggtggcgccaggcgcaacacgcgtg
 16201 gctcgaacttctactgtgggttgcggccggccacccggcgccgtcggtcgccac 16260
 EarI | SacII | BsrFI |
 MspAII | MspAII | BsrDI |
 BstDSI || MmeI |
 gcagcgccgcggaaagagaacctcaacgcggcagccgcggcaatgcagccgggtggaggaca
 16261 cgtcgccgcgccttcttgggttgcggccgtggccgttacgtcggtccacccctgt 16320
 BcgI | BcgI | BcgI | BseRI |
 BbvCI | BbvCI | BbvCI |
 Bpu10I | Bpu10I | Bpu10I |
 BglI | BssHII | BssHII |
 tgaacgatcatggcattcgccggcgcacacccgtccacacgggtgaggagaagcgccgtg
 16321 acttgcttagtacggtaagcgccgtgtggaaacgggtgtggccgactcccttcgcgcgac 16380
 BsiEI |
 EaeI |
 EagI |
 GdIII | FspI |
 MspAII | MspAII | MspAII | AvaI |
 agggccaaacgcggcccgaaagctgcgcggccggccgtgcgcacacccgggtcgagaagcc
 16381 tccgggttcgtcgccggccgtgcacggcgccggccgtgcgtggccgtccaccccttcggag 16440

Figure 28x

58 / 85

Figure 28Y

59 / 85

BcgI
BsiEI
EaeI
EagI
GdIII
BssHII BssHII NotI BmrI BsaHI
accgcgcgcctggggcgcacaaacgcggccgactggcgcaccacccgtcgatgacg
17161 tggcgcgcgggaccccgcgcgtgtttgcgcggcgtgaccgcgtggcagctactgc 17220

XcmI
BcgI BssHII BseRI
ccatcgacgcgggggtggaggaggcgcgaacctacacgcccacgcgcaccagggttcca
17221 ggttagctgcgccacccacccctccgcgcgtgtcgatgtgcggggcgcgggtcacaggt 17280

EaeI BstDSI BanII BsmBI
GdIII XcmI Bsp1286I HaeII EarI
cagtggacgcggccattcagaccgtggtgcgcgaggccccggcgtatgctaaaatgaaga
17281 gtcacctgcgcggtaagtctggcaccacgcgcctggggcgcatacgtttacttct 17340

EciI BglI BcsI
gacggcgaggcgcgttagcacgtcgccaccggccgcacccggcactggccgcacacgcg
17341 ctggccgcctccgcgcacgcgtgcagcgggtggcggcggctggggcgtgacggcgggttgcgc 17400

BsiEI
EaeI
EagI
GdIII EaeI BglI
BsrFI GdIII SfiI BsrBI
cggcgccggccctgctaaccgcgcacgtcgccaccggccgcacggccatggggcc
17401 gccgcgcgggacgaattggcgcgtgcagcgtggccggctggccgggtacgcccggc 17460

SacII MspAII Bsp1286I AhdI
BstDSI EaeI BmgI HaeIVI AfI III
GdIII SacII BseSI Hin4II TaqII
ctcgaaggctggccgcgggtactgtcaactgtgccccccagggtccaggcgcacgcggcc
17461 gagcttccgacccggccataacagtgcacacgggggtccagggtcccggtgcacataaccc 17520

EaeI GdIII SacII MspAII AhdI
BscDSI EaeI BmgI HaeIVI AfI III
ccgcagcagccgcggccattagtgcataactcagggtcgccaggggcaacgtgtatggg
17521 ggcgtcgccgcggtaatcacaactgatggatcccagggtccccgtgcacataaccc 17580

Bsp1286I BmgI BseSI FspI
tgcgcgactcggttagcggccgcgcgtggccgtgcgcacccggccggccgcacactaga
17581 acgcgtgagccaaatcgccggacgcgcacgggcacgcgtggggcggggcgttgc 17640

BssHII BcIVI
MspAII
ttgcaagaaaaactacttagactcgactgtgttatgtatccaggccggggggcga
17641 aacgtttttttgtatgaaactgagcatgacaacatacataggcgccggccgcgt 17700

EarI BpmI BglII BstYI
acgaagctatgtccaaagcgcacaaatcaaagaagagatgtccaggccatcgccggaga
17701 tgccgcatacagggttcgcgttttagttttttccacagggtccaggcagcgcggcc 17760

EarI SapI
tctatggcccccgaagaaggaaagcaggattacaagccccgaaagctaaagcgggtca
17761 17820

Figure 28Z

Figure 28AA

61/85

Figure 28BB

62/85

19141 BbsI BsmBI:
 cgcttggctgttaactatggtagaaatggaaagacatcaactttgcgtttggggggg
 gcgaaaccaggacattgataaaaacatccacccctgttagttgaaacgcagagaccggggc 19200

19201 BanI BsrBI
 EcoRV EcoS7I
 cgacacggcccgccccggatcatggaaaactggcaagatccgcaccagcaatatgagc
 gctgtggcgagcggggcaagttacccttgaccgtttatagccgggttgtataactcg 19260

HaeII BanII
 BsaHI Bsp1286I
 NarI MspAII
 BanII PvuII BanII ApoI
 ggtggcgcccttcagctggggccgtggggggcattaaaaatttcggttccaccgtt 19320
 ccaccggaaagtgcgaccggagcgacacccggccgtattttaaagccaagggtggcaa

BbvCI
 HaeI Bpu10I
 SstI HaeI AlwNI
 aagaacctatggcagcaaggccggaaacagcagcacaggccagatgtggggataagttg
 tctttgataccgtcgccccggacccgtgggttgtgggtctacgactccccattcaac 19380

ApoI HaeI
 MspI HaeI
 aaagagcaaaatccaaacaaaagggtggtagatggccctggcattagcggggtg
 ttttcgttttaaagggtgtttccaccatctaccggaccggagaccgttaaccggccac 19440

HaeI HindIII
 MscI EaeI BglI BtsI
 gtggacacctggccaaaccaggcagtgcaaaataagattaacagtaagcttgcacccgg
 cacctggaccgggtggccgtcacgtttatcttaattgtcattcgaaactggggggga 19500

BpmI BsiEI
 BstDSI BseRII BsaXI
 EaeI Hin4I
 EagI Tth11I
 GdiI BsaXI
 BsrFI Hin4I
 cccgttagaggagcccccacggccgtggagacagtgtccagagggggcgtggcaaaag
 gggcatcttcgtggagggtggccggcacccctgtcacagagggtctccccgcaccgg 19560

Hin4I SunI
 cgtcccgcccccacagggaaagaaacttggtgacgcacatagacgagcccccgtac
 gcaggcgccgggtgtcccccttggagaccactgcgtttatctgtcccgaggagcatg 19620

BstAPI Bsp24I
 HaeI BstDSI
 SstI NcoI
 Bsp24I ScyI
 BplI BseRI XcmI BsaWI
 gaggaggcactaaagcaaggccgtccaccacccgtccatgcgtccatggccaccgg
 ctccccgtatccgtccggacgggtggggcagggttagcgcgggtaccgcgtggcet 19680

BspGI BspMI
 gtgtggccagcacacacccgtaacgtggacctggccctccccccggacacccagca
 cacgaccggccgtgtggcattgcgtggacccgtggacggagggggggggtgtggcgt 19740

TaqII BsiEI
 aaacctgtgtgtccaggccggaccggccgttggtaaccgtccatggcgtggccgtcc
 tttggacacgcggccgggtggccggcaacaacatggcaggatggcggccgcaggac 19800

RsrII BsiEI
 MspAII PvuII F

Figure 28CC

63 / 85

Figure 28DD

64 / 85

Figure 28EE

65 / 85

Figure 28FF

BstDSI SmlI SmrI
 22021 gggcggtttccgcggctgggccttcacgcgccttaagactaaggaaaccccatcactgg
 ccccggaaaaggcgccgaccggaaatgcgcggaaatttgatccccggatggtagtggacc
 Pfl1108I
 BanII
 Bsp1286I
 Avall
 22081 gctcgggtacgacccttattacacctacttggcttataccctaccttagatggAACCT
 cgagcccgtatggaaataatgtggatgagaccgagatacggatggatccacccggat
 HaeI MspAII
 MscI Earl HaeI
 EaeI DrdI Pvull
 22141 ttaccccaaccacaccccttaagaagggtggcattacccttgacttttgtcagctggc
 aaatggagttggatggaaattttccacccggtaatggaaactgagaagacagtgcaccg
 BsrDI HaeII HincII
 22201 ctggcaatgaccgcctgcctaccccaacgagtttgaattaaagcgctcagttgacgggg
 gaccgttactggcgacgaaatggggttgtccaaactttaattcgcgagtcactggccc
 AclI BmrI PflMI DrdII NheI
 22261 agggttacaacgttggccagtgtaacatgaccaaaagactggttccctggtacaaaatggtag
 tcccaatgttgcacccgggtcacattgtactggtttctgaccaaggaccacgttacgatc
 NspI
 TatII
 22321 ctaactacaacattggctaccagggtttatataccagagagctacaaggaccgtatgt
 gattgttgttgcacccgtatggccccgaagatatagggtttctcgatgtttcccggtaca
 Bsp24I Bsp24I
 22381 actcccttttttagaaaccttccagccccatgagccgtcaggtggatgtatccaaataca
 tgaggaagaaaccttgcaggctgggtactcggcagtcaccacccatgttacgatgt
 PflMI BsbI
 22441 aggactaccaacagggtggccatccatcaccaacacaacacttggatggatggctacc
 tccctgttgttgcacccgtatggatgtttttggatggacccaaacaaccgtatgg
 HaeI
 StuI
 22501 ttggccccccatgcgcgaaggacaggccatccctgttaacttccctatccgcctatag
 aacgggggtggatgcgcgtttctgtccggatgggacgtatggatggggataggcgaatattc
 BsiEI
 Pvull
 SgfI BpmI
 22561 gcaagaccgcagtttgcacccatccatccatccatccatccatccatccatccatcc
 cggttgtggcgatgcgtatggcccttttcaaaagaaacgcgtatggggaaaccgg
 BstDSI
 NcoI
 StyI
 22621 gcatcccatccatccatccatccatccatccatccatccatccatccatccatccatcc
 cgtagggtaagaggatccatggaaatccatggatccatccatccatccatccatccatcc
 BstDSI
 BamHI NcoI
 BstYI StyI
 22681 ttcttccatgcgcacccatccatccatccatccatccatccatccatccatccatccatcc
 aagagatgcgggttggggcgatgtactgaaaaactccacccatggatccatccatccatcc
 BsiEI
 EaeI
 EagI

Figure 28GG

67 / 85

Figure 28HH

Figure 28II

69 / 85

Figure 28JJ

70/85

Figure 28KK

71/85

Figure 28LL

72 / 85

Figure 28MM

26581 ----- 26640
 cgttttgaacctccggatacctggcggaaacttgcgcgaggcacccggcggtggaccg
 EcoNI AlwNI
 | |
 26641 ggacatccatttccccgaacgcctgcctaaaaccctgcacacagggtctgccagacttcac
 cctgttagaaaaaggggcttgcggacgaattttggacgttgtcccacacggctctgaagtg 26700
 Bpu10I
 HaeII
 NspI Eco47III
 | |
 26701 cagtc当地ggatgtgcagaactttaggaactttatccatagagcgtttggaaatttggc
 gtcagtttgcataaacgtcttggaaatccctggaaataggatctcgcgagttttttagaacgg 26760
 Bs1HKAI
 Bsp1286I
 BseSI Bsp1286I
 BspMI BmgI | BciI
 AarI ApaLI BseSI BsmI
 | | | |
 26761 cggcacctgtgtgcacttccatgcactttgtgcccattaagtaccggcgaaatggccccc
 ggggtggacgacacgtgaaggatcgctgaaacacggtaattcatggcgacgttacgggagg 26820
 NheI
 PstI
 BtsI SfcI
 | |
 26821 gccgtttggggccactgttacccctctgcagctagccaaactacccctggcttaccactctga
 cggcggaaaccccggtgacgatggaaagacgttgatcggttggaaacggatggtgagact 26880
 BsrBI BsrBI AccI BstAPI
 | | | BpmI
 | | |
 26881 cataatggaaagacgtgagcggtgacggctactggagtgtactgtcgccgtcaacctatg
 gtattacccctctgactcgccactggcagatgacccatgtgacaacgtcgacgttggatac 26940
 BsrBI MspAII BsrBI BanI
 | | | PvuII | |
 | | |
 26941 caccccgcacccgtccctggtttgcatttcgacgtgcctaaacggaaatggaaatccatcg
 gtggggcggtggcgagggaccaacgtttaaggcgatcgacgttgcacgttgcggatac 27000
 EcoO109I MspAII SacI
 Psp5II PvuII BstDSI
 SanDI Ahdi
 | | |
 KpnI SfcI HaeIV Hin4I
 | | | |
 27001 tacctttgagctgcagggcccttgcgttgcacggaaatggccggccgggggttggaaact
 atggaaaactcgacgtcccaggagcgacgttttgcggccggggcccaacttta 27060
 AatII BsaHI ApoI Bsu36I
 | | |
 27061 cactccggggctgtggacgtcggttacccctggcaaaatttgcgttgcggccggcc
 gtgagggccccgacacctgcacggcaatggaaacatggacttgcgttgcgg 27120
 BssSI UbaLI BbsI
 | | |
 | | |
 27121 ccacgagatttagtttgcacggaaatccggcccaatgcggagtttgcgttgcgg
 gttgttgcgttgcggccggggccatgcgttgcggccggcc 27180
 Pfl1108I MunI
 | | |
 HaeI |
 MscI |
 EaeI | |
 BstXI | | |
 | | |
 27181 cgtcattaccaggggccacattttggcaatttgcacggccatcaacaaaggcccccaaga
 gtagtgcgttgcggccggggccatgcgttgcggccggcc 27240
 BmrI BsrBI BanI
 AdhI HaeIV Bsp1286I
 | | | SacI
 | | |
 27240 gtttctgttgcgttgcggccggggccatgcgttgcggccggcc 27240
 Pf1108I

Figure 28NN

74/85

27241 ----- 27300
 caaagacgatgtttcccccggggaaatgaacctggggcaggccgtttcgagtt

ApaI
BamII
Bsp1286I
BmgI
BseSI
EcoO109I
SacII |||
MspAI |||
BseRI |
| cccaatccccccggccggcagccctatcagcagcagccggggcccttgccttcagga
27301 ----- 27360
 gggtagggggggggggcggtcggtatgcgtcggtcgccggaaacgaagggtctt

MspAI
PvuI
PstI
BamI TaqII |||
SfcI ||| BstDSI TaqII BmrI |
| | | |
tggcacccaaaaagaagctcgacgtcgccggccacccacggacgaggaggaaatactggg
27361 ----- 27420
 accgtgggtttttcttcgacgtcgacggccggcggtgggtgccttcctatgaccc

BseRI
BseRI BseRI | BbsI
BseRI | | BmrI |||
acagtcaaggcagaggagggttttggacgaggaggaggacatgtggaaagactgggaga
27421 ----- 27480
 tggtagtcgtctcccaaaccgtctccctccctctgtactaccctctgaccctt

HindIII
EcoNI | EarI | TaqII BcgI
| | |
gcctagacgagggaaatcccgagggtcgaaagagggtgtcagacgaaaaccgtcaccctcg
27481 ----- 27540
 cggatctgtccctcgaaaggctccagtttccacagtgtttgtggcagtggagcc

HaeII
BsaHI |
NarI |
BanI |
BsrFI ||| BsaWI
BsmI NgoAIV ||| BsrFI
BsiEI | SgrAI ||| BcgI PinAI BseRI
| | | |
tcgatttttttcggccggccccagaaatcggaaccggttccagcatggctacaacct
27541 ----- 27600
 agcgtaaaggggagcggccgggggtttttagccgtggcaaggctcgataccgtttggaa

BsrFI
NgoAIV
HaeII
BsaHI |
Bsu36I |
BsrBI | NarI |
BsaXI | BanI | BtsI
| | | |
ccgctccctcaggcgccggcactggccgttgcggacccaaccgttagatggacacca
27601 ----- 27660
 ggcgaggagtcccgccggccgtgacggcaagcggctgggttggcatctaccctgtgg

BsrFI
HgiEII
DrdII ||| Tth111II
| | |
ctggaaaccaggccggtaagtccaaagcagccggccgttagccaaagagcaacaacac
27661 ----- 27720
 gactttgtcccgccatctagggtcgccggccgtatccgggttctcggtgtgg

Tth111II
Bsp1286I | |
BmgI | |
BseSI | | | RleAI
| | |
gccaaggctaccgctatggcgccggcacaagaacgcatagttgcttgcgttgc
27721 ----- 27780
 cggcccgatggcgagttaccggccgttttgcgttatcaacgaacgaacgttctga

HaeI
DraII |
| |
gtggggggcaacaccccttttgcggccgggttttttgcgttgcgttgcgttgc
27781 ----- 27840
 caccgggttgcgttagaggaagcggccggcggaaagaagatggtagtgcggcaccggaaagg

Figure 2800

75/85

Figure 28PP

	SfcI	BsrFI		
	BsgI	SgrAI	MspAII	
27841	cccgtaacatcctgcattactaccgtcatctctacagccccatactgcacggggggcagcg			27900
	gggcattgttaggacgtaatgatggcagtagagatgtcggtatgacggccgcgcgc			
	EaeI	BsiEI		
	GdIII	BsaWII		
27901	gcagcggcagcaacacagcagcggccacacagaagcaaaggcgaccggatagcaagactctg			27960
	cgtcgccgtcggtcgtcgtcgccgtgtgtttcggtcggtatcggtcgagac			
	BsaHI			
	NarI			
	BanII			
	BseRII			
	BseRI			
	HaeII			
	Eco47III			
27961	acaaagccccaaagaaatcccacagcggcggcagcagcaggaggaggcgctgcgtcg			28020
	tgttgcgggttttttaggtgtcgccgcgtcgctcgctttttcgccatcggtcgac			
	HaeII			
	AceIII	BsaI		
28021	cccaacgaaccgtatcgaccgcgagcttagaaacaggatccccactctgtatgt			28080
	gggttgcttgggcatacgctggcgctcgaaatttgtctaaaaagggtgagacatacga			
	AceIII	BsaI		
28081	atatttcaacagcaggggccaagaacaagagctgaaaataaaaaacaggctctgcga			28140
	tataaagtgtctcgccccgtttttgtttctcgactttttatttttgtcgagacgt			
	MspAII	Eco57I		
	PvuII			
28141	tccctcacccgcagctgcctgtattcacaaaagcgaagaccagctcgccgacgcggaa			28200
	agggagtggcgctcgacggacatagtgttttcgttctagtcgaagccgcgcac			
	BbsI	AflII		
	EarI	SmlI		
	BssHII	SpeI		
28201	gacgcggaggcttcttcagtaataactgcgcgtgacttttaaggaccgtttcgcc			28260
	ctgcgcctcgagagaagtcattatgacgcgcactgagaatttcgtatcaaagcgg			
	HaeII			
	BsaHI			
	EaeI	NarI		
	GdIII	BanII		
	ApoI	Bsp24I		
	BpmI	BsaXI		
28261	ctttctcaaaatttaagcgcgaaaactacgtcatctccagcggccacacccggccagca			28320
	gaaagagtttaattcgcgcttttgcgcgttgcgcgtcgccgggtgtggccgcggcgt			
	NspI			
	Bsp24I	AfI		
AlwNI	HaeII	ApoI	BspLU1I	
28321	cctgtcgctagcgcattatgagcaaggaaatcccacgccttacatgtggagttaccag			28380
	ggacagcgtcgcgtaataactcgtttttaagggtgcgggatgtacacctcaatggc			
	AceIII	BpmI		
28381	ccacaaaatgggacttgcggctggagctgcaccaagactactcaacccgaaataactacatg			28440
	ggtgttttacctgaacgcgcacccgcacgggttgcgtatgcgttgggttatttgcgtac			
	HincII			
	RleAI			
	EcoO109I	SmaI		
	Psp5II	AvaI		
	SandI	EcoRV		
			ApoI	
			ECORI	
28441	agcgcgggacccacatgatattccgggtcaacggatccgcggccacggaaacccgaaatt			28500
	ccgcgcctgggtgtactataggcccagggtttcgccataggcgccgggtttggcttaa			
28501	cttttggaaacaggcggttattaccaccacacccgtataaaacccatatccccgtatgtgg			28560

76 / 85

Figure 28QQ

77 / 85

Figure 28RR

78 / 85

Figure 28SS

79 / 85

Figure 28TT

Figure 28UU

81/85

BseRI
Bsu36I | DrdII
32281 tacacacttccctcaggattacaagctcccccgcgtttagaaccatatacccaaggaaacaaccc
atgtgaaggagtcctaatgttgcaggaggcgcaatcttggatagggcccccttgggg 32340

PstI
BtsI | BbsI
SfcI | RleAI | BsaAI DrdIII
32341 attccctgaatcagcgtaaaatcccacactgcaggaaagacctcgcaactcacgtgt
caaggacttagtcgcattttaggtgtgacgtcccttctggagcggtgcattgagtgcaca 32400

MspAII
BpmI
32401 gcattgtcaaagtgttacattcgggcagcagcggatgaaaaaccatggtagcgcggg
cgttaacagtttccaaatgttaagccgtcgccctacttaggaggccataccatcgcccc 32460

AccI
32461 tttctgtctaaaaggaggtagacgatccctactgtacggagtgcggcagacaaccgag
aaagacacagtttccatctgttagggatgacatgcctcacgcggctctgtggctc 32520

Pfl1108I
BsbI | Tth11II
32521 atcggttgtggtcgttagtgcatagccaaatggaaacgcggcgttagtcataatccctgaag
tagcacaaccaggcatcacagtaggtttcacccgcggccgtcattcgtataaaggacttc 32580

BsmBI
SexAI Eco57I BglII BsaWI |
BstYI Tth11II | BsAI
32581 caaaaccagggtgcggcgtgacaaacagatctgggttccgggttgcggcttagatcgc
gttttgtccacgcggcactgtttgtctagacgcagaggccagagcggccgaaatctagcg 32640

HaeII
XcmI
BsaWI
NarI
BanI
32641 tctgtgttagtagttgttagtatccacttctcaaagcatccaggccccctggcttcg
agacacatccatcaacatcatataggcgagagaatcttgcgttaggtccgcggggaccgaac 32700

MspAII
32701 gttttatgtaaaacctccatgcgcgcgtgcgcctgacaaacatccaccaccgcagaataa
ccaagatacatttgaggaagtacgcggcagggactattgttaggtggcgccatt 32760

BsrBI
EarII
SapI
TaqII | AceIII ||
32761 gccacacccaaaggccaaacctacacatcggtctgcgagttcacacacggggaggagcggaaaga
cggtgtgggtcggtggatgtcaagcaagacgcgtcaagtgtgtgccttcgccttct 32820

BseRI DrdII | BglII
32821 gctggaaaggaaaccatgtttttttttatccaaaagattatccaaaacctcaaaatgaag
cgacctttggtacaaaaaaaaataaggttttctaataggtttggagttttacttc 32880

BsaWI | SfcI
32881 acctattaagtgaacgcgtccccctccggtggtggtccaaacttcacagccaaagaaca
tagataattcacttgcgcgaggggaggccacccgcaccatgtttggatgtcggtttttgt 32940

Eco57I HgiEII |
32941 gataatggcatttgcgttaagatgttgcacaatggttccaaaaggcaaacggccctacgtc
ctattaccgtaaaacatccacacgtgttaccgaaggtttccgttgcggggagtgcag 33000

BseRI
32981 caagtggacgtaaaggctaaacccttcagggtgaatctccctataaaacattccagcacc

Figure 28VV

33001 ----- 33060
 gttcacctgatttccgatggaaagtcccacttagaggagatttgtaaaggcggtgg
 33061 ----- 33120
 ctcacccatgccccaaaataattttcatctcgccacccctcaatataatccaaagccaaacc
 aagttggtagccccatccaagagtagagccggcgaaagagttatcatagagatccgttttag
 BpmI
 SspI EaeI GdII Eco57I HaeII Bce83I SmlI
 | | | | | | |
 33121 cccgaatattaagtccggccattgtaaaaatctgtccagagcgcctccacccctcagccct
 ggcttataattcaggccggtaacatccatagacgaggctcgccggaggtggaaagtcggaa
 Tch111II RcaI ApoI
 33181 caaggcgaattcatgattgaaaaatccaggtttttcacagacctgtataagattaaaa
 gtttgtcgcttagtactaacgtttttaaagtccaaaggagtgtcgacatattttaaagttt
 EcoO109I PspSII MspAII BsgI PvUII BspMI
 33241 agcggAACATTAACAAAAATACCGCGATCCCGTAGGTCCCCCGCAGGGCCAGCTGAACA
 tcgcctttgtattgtttttatggcgcttagggcatccaggaaaggcgccccggacttgr
 EaeI GdII BsgI
 MsI I
 33301 caatcgtaggtctgcacggaccaggcgccccactcccccggtaggaacccggacaaaa
 attagcacgtccagacgtggctggcgccggtaaaggggcggtttttggaaactgtttt
 RleAI
 33361 gaacccacactgattatgacacgcataactcgagctatgttaaccagggttagccccatg
 ctgggtgtgactaatactgtcgctatgagccctcgatcacgttttttttttttttttt
 HindIII
 |
 33421 taagcccttgcacggccggatataaaatgcaagggtgtgtccaaaaatcaggcaa
 attcgaaacaacgttcccgccctatattttacgtttccacgacgattttttttttttttt
 Pfl1108I MsI BspMI
 33481 agcctcgccaaaaaagaaggcacatcgtagtcatgtcatgcagataaaaggcggttaag
 tcggagcgccgttt
 BsaWI BspEI OrdII NspI
 | | | | | | |
 33541 ctccggAACCCACACAGAAAAAGACACCACTTTCTCTCAAACATGTTGGGGTTCTG
 gaggcccttgggggttt
 DraI
 33601 cataaacacaaaaataaaataaaaaacatccaaacatttttttttttttttttttt
 gtatgttt
 BglI EaeI BsrFII
 GdII NgoAIVI
 | | | | | | |
 33661 ggaaaaacaacccataaagcataagacggaccacggccatggcggtggaccgtaaaaa
 cccccccgttt
 BstEII TaqII BsaWI
 | | | | | | |
 33721 aactgggtcaccgtgataaaaaaggcaccacccgacagcttttttttttttttttt
 ttgaccaggccactaatt
 TaqII BsaEI
 33781 atgttaagactcggttt
 tacatt
 33840

Figure 28WW

83 / 85

Enzymes that do cut:

AarI	AacII	AccI	AceIII	AclI	AfI III	AfI III I	AhdI
AlcI	AlwNI	Apal	ApalI	ApoI	Ascl	Aval	AvrII
BaeI	BamHI	BanI	BanII	BbsI	BbvCI	Bce83I	BcgI
BcIV	BclI	BglI	BglII	BmgI	BmrI	BplI	BpmI
Bpu10I	Bpu1102I	BsaI	BsaAI	BsaBI	BsaHI	BsaWI	BsaXI
BsbI	BseRI	BssI	BsgI	BsiEI	BsiHKA I	BsmI	BsmB I
Bsp24I	Bsp1286I	BspEI	BspGI	BspLU11I	BspMI	BsrBI	BsrDI
BsrFI	BsrGI	BssHII	BssSI	BstAPI	BstDSI	BstEII	BstXI
BstYI	BstZ17I	BsuJ6I	BtsI	ClaI	DraI	DraIII	DrdI
DrdII	EaeI	EagI	EarI	EciI	Eco47III	Eco57I	EcoNI
Eco0109I	EcoRI	EcoRV	FseI	FspI	GdIII	HaeI	HaeII
HaeIV	HgiEII	HindI	HincII	HindIII	HpaI	KpnI	MluI
MmeI	MscI	MslI	MspAll	MunI	Nari	NcoI	NdeI
NgoAIV	NheI	NotI	NruI	NsII	NspI	PacI	Pfl1108I
PflMI	PinAI	Pmel	PmlI	PshAI	PspSII	PstI	PvuI
PvuII	RcaI	RleAI	RsrII	SacI	SacII	Sali	SanDI
Sapi	SbfI	Scal	SexAI	SfcI	SfiI	SgfI	SgrAI
SmaI	SmlI	SnaBI	SpeI	Sphi	SrfI	Sse8647I	SspI
StuI	StyI	SunI	Swal	TaqII	TatI	Tth1111I	Tth1111I
UbaLI	VspI	XbaI	XcmI	Xhol	XmnI		

Enzymes that do not cut:

Navy

Figure 28xx

84/85

O A 1 2 3 4 5 - Days p.i.
[REDACTED] = RID β

[REDACTED] = 14.7K

[REDACTED] = gp19K
a b c d e f g

Figure 29

14.7K
RID β
gp19K

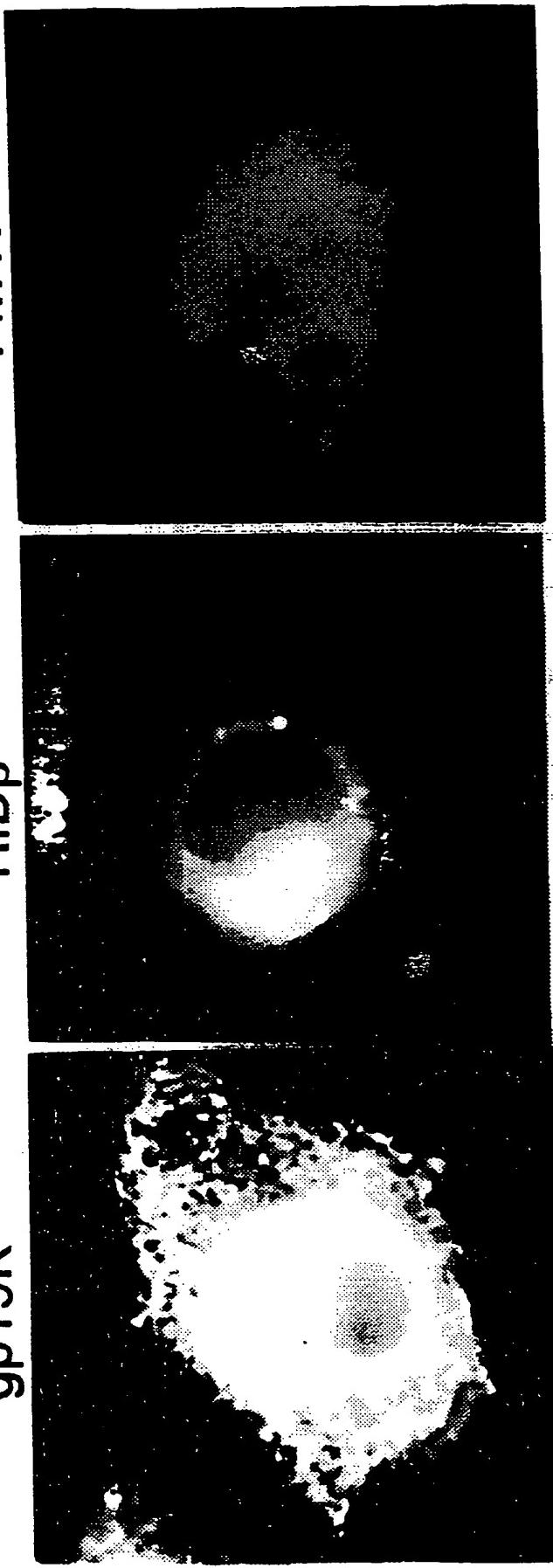


Figure 30A

Figure 30B

Figure 30C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14239

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) C12N 7/01, 15/34, 15/87
US CL 435/456, 372, 377

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/456, 372, 377

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, CANCERLIT, WPIDS, USPATFULL
apoptosis, FAS, TNFR, DR3, TRAIL-R1, TRAIL-R2, leukocyte, transplant, adenovirus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,674,734 A (LEDER et al) 07 October 1997, see entire document.	1-25
Y	GOODING, L.R. et al. The anti-viral activities of TNF: Adenovirus strikes back. J. Cell Biochem. 27 January-03 February 1990, Suppl. 0, Vol. 14, Part B, page 18, abstract CD34.	1-25
Y	GOODING, L.R. et al. Adenovirus genes that modulate the sensitivity of virus-infected cells to lysis by tumor necrosis factor. J. Cell. Biochem. 08 February 1992. Suppl. 0 Vol. 16, part B, page 269, abstract J005.	1-25

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

20 AUGUST 1998

Date of mailing of the international search report

08 OCT 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Faxsimile No. (703) 305-3230

Authorized officer

MARY TUNG

Telephone No. (703) 308-0198

10 126

INTERNATIONAL SEARCH REPORT

International application No. PCT/US98/14239

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	ZHANG, H. et al. Amelioration of collagen-induced arthritis by CD95 (Apo-1/Fas)-ligand gene transfer. J. Clin. Invest. October 1997, Vol. 100, No. 8, pages 1951-1957, see entire document.	1-25